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THE

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SCIENCE BULLETIN

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THE UNIVERSITY OF KANSAS
SCIENCE BULLETIN

VOL. XVI.] MARCH, 1926. [NO. 1.

Antigenic and Metabolic Studies of *Bacillus typhosus*.*

CORNELIA M. DOWNS, Department of Bacteriology.

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* Submitted to the Department of Bacteriology and the Faculty of the Graduate School of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

INTRODUCTION.

RELIABLE methods for the identification and classification of bacteria have been eagerly sought by many investigators. Kendall (1923) says, "We are not so much interested in what bacteria are as in what they do." Morphological classification has been largely superseded by physiological classification. It has been found that the cultural reactions of bacteria are fairly stable and that most organisms can be divided into groups by means of these reactions. As the science of immunology has developed it has been found that the members of these groups, although giving identical cultural reactions, can be separated into subgroups by means of serological tests. A case in point is that of the pneumococcus group. All pneumococci give certain definite cultural reactions, but serological tests separate these apparently identical organisms into four sharply defined groups. Of late years studies on streptococci, staphylococci, *B. dysenteriæ*, *B. diphtheriæ* and others have shown that organisms may be grouped by means of cultural reactions and these groups further subdivided by serological reactions.

The stability of these cultural and serological reactions has an important bearing on their use in the identification of organisms. Variations in physiological and antigenic properties of many organisms have been reported.

The typhoid group has usually been considered homogeneous in cultural and antigenic reactions, and if this were true it would seem to differ sharply from the majority of other organisms. Enough variation, however, has been reported by Hooker (1916), Weiss (1917), Teague and McWilliams (1919), Rotky (1921), and others, to give point to an intensive study of several strains of *B. typhosus*. This work was accordingly undertaken.

Part I deals with a study of the antigenic differences exhibited by strains of *B. typhosus* and the relation of such antigenic differences to any cultural peculiarities present.

Part II deals with the effect of hydrogen-ion concentration on the metabolism of these same strains; their dextrose metabolism and viability, and the correlation of these reactions with their antigenic characters.

PART I.
CULTURAL AND SEROLOGICAL REACTIONS.

EXPERIMENT 1.—CULTURAL TESTS.

METHODS: Thirty-two strains of *B. typhosus* were collected from as widely separated sources as possible. The source, place of isolation, name and date are shown in Table I. Preliminary cultural reactions were determined on each strain. The carbohydrate medium was Hiss' semisolid medium, to which was added 1 per cent of the carbohydrate desired and Andrade indicator to make a pale flesh color when cold. A duplicate set of determinations was made, using meat-infusion broth, adjusted to pH 7.0, to which 1 per cent of the carbohydrate was added, litmus being used as an indicator. For the lead-acetate agar 1 cc. of 1 per cent lead-acetate solution was added to the semisolid medium. To test for liquefaction and gas production on noncarbohydrate medium, a 2 per cent peptone gelatine, made according to Treece (1920), was used. The gram strains were made from 24-hour meat-extract broth cultures, and also from 24-hour agar slants.

TABLE I.—Strains of *B. typhosus* used.

1	Blood culture, Lawrence, Kan.	57	1913
21	Blood culture, Kansas City, Mo.		1919
23	Blood culture, University of California.		1914
25	Blood culture, Johns Hopkins Hospital.		
27	Blood culture, Kansas City, Mo.	Nelson	1919
36	Blood culture, Lawrence, Kan.	Brownbeck	1920
40	Blood culture, Lawrence, Kan.	Clements	1921
4	Feces, Lawrence, Kan.	Smith	1919
5	Feces, Lawrence, Kan.	Hunter	1919
6	Feces, Lawrence, Kan.		1918
8	Feces, Lawrence, Kan.		1919
16	Feces, carrier, Beau Desert, France.		1918
20	Feces, Topeka, Kan.		1919
24	Feces, fatal case, Johns Hopkins Hospital.		1919
30	Feces, carrier, Michigan.	Dardrich	1920
31	Feces, carrier, Michigan.		1920
32	Feces, carrier, Michigan.	Dowd	1920
33	Feces, carrier, Michigan.		1920
34	Feces, carrier, Michigan.		1920
39	Feces, case, Lawrence, Kan.	Hess	1921
44	Feces, case, Lawrence, Kan.		1921
7	Spinal fluid, Halstead, Kan.		1919
12	Spleen, autopsy.	Rawlings	
15	Gall bladder, autopsy, France.	Wahle	1918
2			
3		Bender	
10	No history, New York Board of Health.	Mt. Sinai	
11		Pfeiffer	
13	American Museum.	Hopkins	
14	American Museum.	Miller	
17	Institute of Berlin.	Ebert	1888
19	University of Chicago.	Jordan	1889

RESULTS.

Table II shows that the majority of the cultural reactions were typical. Three strains—Nos. 21, 40, and 12 (the Rawlings strain)—fermented xylose slowly. Weiss (1917) has reported that the Rawling's strain does not ferment xylose. Teague (1920) considered this strain to be a slow fermenter. The litmus milk was kept six weeks before being discarded. The milk remained a permanent lilac color in all except six organisms. Five turned back to neutral in three weeks and one became a deep blue color after one week. Three strains gave slight acidity in salicin and two strains were negative in dextrin. Krumwiede (1918) reports fermentation in dextrin as varying with the sample used. The two cultures giving an alkaline reaction in dextrin might, therefore, have shown typical acid production in another sample. My strains were uniformly negative on dulcite and arabinose. Teague (1920) reported that eleven out of forty-one strains of *B. typhosus* fermented these sugars slowly. In addition to the above strains an organism isolated from the feces of a clinical case of mild typhoid was studied. This organism is designated as No. 5. The patient at no time gave a positive Widal. The organism differed culturally from *B. typhosus* in that it blackened lead acetate slowly, did not ferment xylose nor dextrin, fermented rhamnose, and gave a distinct alkaline reaction in litmus milk after seventy-two hours, but with no saponification.

The danger of confusing non-gas-producing paratyphoid strains with *B. typhosus* has been emphasized. Ten-Broeck (1916) reports a non-gas-producing hog-cholera bacillus which resembled in some respects *B. typhosus*. Krumwiede (1918) also reports a similarity in cultural and serological reactions between *B. pullorum* and *B. sanguinarium* and *B. typhosus*. Myers (1920) reports the isolation from a clinical case of typhoid of a rhamnose-positive *B. typhosus* which was also atypical in its serological reactions. It was difficult to decide, therefore, whether No. 5 was a true but irregular *B. typhosus* strain or a non-gas-producing paratyphosus. Krumwiede* kindly examined this strain, and using the fermentation of rhamnose as the deciding factor between the typhoid and paratyphoid groups, considered it a member of the latter group.

* Personal communication from Dr. Charles Krumwiede.

TABLE II.—Cultural characteristics.

NUMBER.	Motility	Gram	Gelatine	Indol	Lead acetate	Dextrose	Mannite	Maltose	Lactose	Saccharose	Dextrin	Rhamnose	Salacin	Dulcite	Arabinose	Xylose	Litmus milk.
1, 2, 3, 4, 6, 7, 8	|	|	|	|	+	+	+	+	|	|	+	|	+	|	+	1 neutral after 1 week.
9	|	|	|	|	+	+	+	+	|	|	+	|	+	|	+	———
10, 11, 13, 14, 15, 16, 17, 20	|	|	|	|	+	+	+	+	|	|	+	|	+	|	+	15, 17, 19 neutral after 3 weeks.
12	|	|	|	|	+	+	+	+	|	|	+	|	+	|	+	———
21	|	|	|	|	+	+	+	+	|	|	+	|	+	|	+	Acid after 10 days.
23-25	|	|	|	|	+	+	+	+	|	|	+	+	+	|	+	23, 24, 26 neutral after 3 weeks.
24, 28, 29, 30, 31, 32, 33, 34, 35	|	|	|	|	+	+	+	+	|	|	+	|	+	|	+	———
26, 27	|	|	|	|	+	+	+	+	|	|	+	|	+	|	+	———

EXPERIMENT 2.—ANTIGENIC DIFFERENCES EXHIBITED BY DIFFERENT STRAINS OF B. TYPHOSUS.

Antigenic irregularities in *B. typhosus* have been noted by Stober (1904), Teague and Torrey (1907), Sheller (1909), Muller (1911), Raubitschek (1913), and Moon (1914). Recently Hooker (1916) and Weiss (1917) have offered a tentative grouping of typhoid strains based on antigenic differences. The occurrence of antigenic differences among several strains of *B. typhosus* used in diagnostic work in this laboratory had been noted from time to time and these differences served to indicate the advisability of an investigation of the serological characteristics of a number of strains of *B. typhosus*. The serological work herein reported consisted of a study of agglutination and absorption tests and Widals using the organisms given in Table I.

AGGLUTINATION TESTS.

METHODS. Eleven sera were prepared by the intravenous injection of killed cultures of *B. typhosus* into rabbits. This was supplemented by the injection of living cultures after several doses of killed cultures had been given. The titre of these sera ranged from 1-500 to 1-15,000. In addition, five sera were obtained from commercial laboratories: Parke-Davis serum, titre 1-10,000; Wichita laboratory, 1-400; University of Chicago, 1-10,000; Lederle (Rawlings, monovalent serum), 1-8,000; New York city board of health, 1-80,000 (bivalent serum prepared from the Mt. Sinai strain, and a freshly isolated strain). All agglutination tests were performed with living cultures washed from a 24-hour agar slant with sterile saline to make an even emulsion. Equal quantities of the suspension and the designated dilution of serum were added to clean test tubes. These tubes were incubated two hours in a water bath at 37° C., and readings were taken. Final readings were taken after twelve to fourteen hours in the ice box.

ABSORPTION TESTS.

The absorption tests were performed as follows: The serum to be absorbed was diluted to one-tenth its titre. This dilution was then saturated with the organisms washed from a 24-hour agar slant to make a heavy emulsion. This was incubated at 37° C. for four hours, and for four days at ice-box temperature, more organisms being added as the supernatant fluid became clear. This length of time was found to be necessary to give complete absorption. A control of diluted serum alone, without organisms, in every case gave a

good agglutination in spite of the prolonged incubation. A second control of the diluted serum, absorbed with the strain used for immunization, was used to determine if absorption were complete. We found the following technical points must be considered in any absorption test:

1. Weight of suspension.
2. Dilution of serum.
3. Time of absorption.
4. Repeated saturation.
5. Temperature.
6. Adequate controls.

Krumwiede (1918) recommends a proportion of one to four or three, or at most one to two, of packed cells to supernatant fluid. Our proportion after the final centrifuging was one to three. It was found that a dilution of one-tenth the titre of the serum was perfectly satisfactory. Although higher dilutions could be used, a lower dilution did not give complete absorption. Three or four hours was not long enough to give complete absorption and frequently absorption was not complete in twenty-four or forty-eight hours. After a standard of four days was chosen, no more trouble was experienced. It was always necessary to add more organisms as the supernatant fluid became clear. The greater the tendency to agglutinate, the larger the number of organisms necessary for complete absorption. The serum being absorbed had to be kept at ice-box temperature because of the well-known tendency of diluted serum to deteriorate at room or incubator temperature. A control of diluted serum which had been incubated under the same conditions as the test serum was necessary to determine whether a drop in titre had occurred, and a control of the serum to be tested saturated with homologous organisms indicated the completeness of the absorption.

WIDAL REACTIONS.

With the exception of a few microscopic tests for comparative purposes, the macroscopic test was used exclusively. Material was usually received, as drops of dried blood on glass and metal slides, or on filter paper. A few Widals were made from clear serum obtained in Wright's capsules. A dilution of 1-25 and 1-50 was made in saline and centrifuged when necessary. It was noticed that fresh serum drawn from the clot and used within twenty-four or forty-eight hours gave a positive agglutination with a larger number of organisms than did the Widals run with dried blood. When dried blood was used a precipitate was usually present in the tubes, giving a positive reaction. This might be due to the presence of hemoglobin, foreign substances on the metal slides or paper, some change in

reaction or biochemical change. No precipitation was noted in Widals where clear serum was used nor in the agglutination tests made with rabbit serum. An equal amount of an emulsion of organisms was added to the serum dilution, doubling the original dilution. All Widals were set up using strains Nos. 1, 2, 3, 10 and 12. The Rawlings strain, No. 12, was selected because it is widely used in the preparation of vaccines and for Widals. Nos. 1, 2 and 3 were used because of certain serological irregularities which they exhibited, and No. 10 because it was an organism giving a clear adherent agglutination with most sera used.

RESULTS.

Table III and IV give a summary of the agglutination reactions with immune sera. Table III shows that the same serum gave varying titres when different strains of *B. typhosus* were used as antigen. Table IV shows these differences even more clearly. From these results we have placed the organisms tentatively into three groups. Group I was made up of eleven organisms, group II of twelve organ-

TABLE III.—Quantitative variations in agglutinations with commercial sera.

NUMBER.	Sera used.					
	Parke-Davis.		Wichita.		University of Chicago.	
	Titre.	Reaction.	Titre.	Reaction.	Titre.	Reaction.
1	1–50	—	1–50	.....	1–8,000	4+
2	1–10,000	3+	1–50	1+	1–10,000	4+
4	1–1,000	1+	1–400	4+	1–2,000	4+
6	1–2,000	4+	1–400	.....	1–4,000	4+
50	1–50	—	1–50	.....	1–50	.....
51	1–50	—	1–50	.....	1–50	.....
52	1–50	.....	1–50	.....	1–50	.....
20	1–4,000	4+	.....	.....	1–8,000	4+

isms; group III of two organisms. Group I serum agglutinated all other organisms in this group in dilutions practically as high as it agglutinated the homologous organism. Group I serum also agglutinated group II organisms, but in lower dilutions. Conversely, the group I organisms are agglutinated by group II serum, but in lower dilutions than were the group II organisms. These two groups were closely related and interagglutinated to the degree indicated in the table. Groups I and II serum gave slight or no agglutination

with group III organisms. Group III consisted of two strains, Nos. 2 and 3, which interagglutinated at 1-12,000, but this high-titred serum agglutinated members of groups I and II in low dilutions or not at all. The results of agglutination tests using the New York horse serum indicated that the same antigenic differences were present, but the titre of the serum was higher. To illustrate: No. 12, Rawlings', was completely agglutinated to 1-80,000, and No. 1 to 1-5,000.

It will be seen by inspection of Table V that the absorption tests showed a closer relationship between groups I and II than did the agglutination tests, strain No. 1 being somewhat more irregular than the other strains. The absorption tests showed a more striking difference between the two organisms in group III and the other groups. The antigenic differences shown by these organisms could not be correlated with their age as with Hooker's (1916) organisms nor with cultural differences as with Weiss' (1917). The antigenic differences between the strains shown by the use of immune serum were also apparent from the results of the Widal tests. If anything, these antigenic differences between the strains were exaggerated.

Table VI gives the results of the Widal tests. From this table it is readily seen that different organisms with the same sera set up at the same time showed marked differences in agglutinability. This may be due to the different agglutinogenic properties inherent in the organisms themselves. Such marked differences probably would not be noted had absorption tests been possible. It is recognized that these twenty positive Widals are too few to provide a basis for accurate conclusions. It seems highly probable that the dried blood method exaggerates the antigenic differences between the organisms, changing what is probably a quantitative into an apparently qualitative difference. The low percentage of positives with Nos. 2 and 3 might be expected from the results given in the absorption tests using immune serum. No. 10, on the other hand, gave a very low percentage of negatives. Those read as partial agglutination would, in clinical work, be called positive. The tubes read as positive gave complete clearing of the supernatant fluid; those read as partial agglutination showed unmistakable agglutination, but with some cloudiness of the supernatant fluid. No. 10, therefore, gave ninety-three per cent positive. No. 12, while giving the highest percentage of complete agglutinations, gave only ninety per cent positive when partial agglutinations are included. It seems probable in view of the results obtained that it might be worth while to use more than one

TABLE IV.—Agglutination reactions with immune sera.

STRAIN.	Serum. 1		4		7		20		12-Lederle.		2		3	
	Titre.	Reaction.	Titre.	Reaction.	Titre.	Reaction.	Titre.	Reaction.	Titre.	Reaction.	Titre.	Reaction.	Titre.	Reaction.
1	1–2000		1–1000		1–500	3+	1–2000	4+	1–2000	3+	1–100	4+	1–100	+
4	1–2000		1–1000		1–500	4+	1–1000	3+	1–2000	4+	1–100	+	1–100	±
6	1–2000		1–500		1–500	5+	1–2000	3+	1–5000	3+	1–100	+	1–100	±
7	1–1000		1–1000		1–3000	4+	1–3000	3+	1–8000	3+	1–100	2+	1–100	3+
8	1–200		1–500		1–3000	4+	1–2000	3+	1–2000	3+				
20	1–1000		1–1000		1–500	3+	1–3000	4+	1–2000	3+				
21	1–1000		1–500		1–2000	3+	1–2000	3+	1–2000	3+				
23	1–200		1–500		1–1000	3+	1–1000	3+	1–8000	3+			1–200	3+
24	1–1000		1–500		1–3000	3+	1–3000	4+	1–8000	4+			1–100	2+
25	1–50		1–1000		1–1000	3+	1–3000	4+	1–5000	3+			1–200	3+
26	1–500		1–2000		1–3000	4+	1–3000	4+						
27					1–1000	4+			1–1000	4+				
28					1–1000	4+	1–1000	4+	1–5000	4+			1–50	3+
29					1–500	4+	1–1000	4+	1–5000				1–50	2+
11	1–200		1–500		1–1000	3+	1–500	3+	1–8000	4+	1–200	4+	1–100	4+
9	1–2000		1–2000		1–2000	3+	1–3000	3+	1–5000	3+	1–1000	3+	1–1000	3+
12	1–1000		1–2000		1–2000	4+	1–2000	4+	1–8000	3+				
10	1–1000		1–2000		1–500	3+	1–2000	3+	1–5000	3+	1–200	3+	1–1000	3+
13	1–1000		1–1000		1–200	3+	1–2000	3+	1–5000	1+	1–200	4+	1–200	3+
14	1–1000		1–2000		1–1000	3+	1–1000	3+	1–2000	3+	1–200	3+	1–1000	3+
15	1–1000		1–500		1–2000	3+	1–2000	3+	1–500	3+	1–100	3+		
16	1–1000		1–5000		1–5000	3+	1–3000	3+	1–5000	1+	1–100	2+	1–100	1+
17	1–1000		1–5000		1–1000	3+	1–300	3+	1–5000	3+	1–100	2+		
19	1–1000		1–1000		1–1000	3+	1–1000	3+	1–5000	1+	1–100	2+	1–100	±
30					1–1000	1+	1–1000	1+	1–8000	4+	1–50	3+	1–50	3+
31					1–400	4+	1–1000	4+	1–8000	4+	1–50	±	1–50	2+
32							1–100	1+	1–5000	3+			1–50	3+
2	1–200		1–500		±		±		1–50	3+	1–15000	4+	1–12000	3+
3	1–200				+		+		1–50	3+	1–15000	3+	1–12000	4+

TABLE V.—Absorption tests with immune sera.

Absorbing antigen.	Sera used.									
	1	12	9	2	3	27	7	13	20	8
1	+	±	±	±	—	±	±	+	±	±
4	+	+	+	—	—	+	+	+	+	+
6	±	+	±	—	—	—	+	+	.....	+
7	±	+	+	—	±	+	+	+	+	+
8	±	+	+	—	—	—	+	+	+	+
20	±	±	+	.....	—	+	+	+	+	+
21	+	+	±	—	—	+	+	+	+	+
23	+	±	+	±	±	+	+	+	+	+
24	+	+	+	—	—	+	+	+	+	+
25	+	±	+	—	—	+	+	+	+	+
26	+	+	±	—	—	+	+	+	+	+
27	+	—	+	—	—	+	.....	+	+	+
9	+	+	+	—	—	+	+	+	+	.....
10	+	+	+	—	—	+	—	±	+	±
11	±	+	+	—	—	+	—	+	—	—
12	±	+	+	±	—	±	—	+	+	+
13	+	+	+	—	—	±	±	+	±	+
14	+	+	±	+	+	±	.....	+	+	+
15	±	—	+	—	.....	±	+	+	+	+
16	+	+	+	—	.....	+	+	+	+	+
17	+	±	+	±	—	+	+	+	—	+
19	+	+	+	—	.....	+	+	+	+	+
2	—	±	—	+	+	—	—	—	—	—
3	—	±	—	+	+	—	—	—	—	—

+ Absorption complete.
± Absorption incomplete but reduction of titre.
— No absorption.

TABLE VI.—Widal reactions.

ORGANISMS.	Sera.																			Percentages.			
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	+	±	—
1	—	—	—	—	+	—	—	....	+	+	±	—	—	+	+	+	+	+	—	±	42	10	47
2	—	±	+	—	—	±	±	—	+	—	—	±	—	+	—	+	±	—	—	—	20	25	55
3	—	....	+	±	±	±	+	—	+	....	—	—	+	+	±	+	±	—	—	—	33	27	38
10	+	....	+	+	+	+	+	....	+	....	±	±	±	±	—	±	+	+	+	+	70	23	6
12	+	+	+	+	+	+	—	+	+	+	+	—	±	+	+	±	±	+	+	+	75	15	10

+ Clear-cut, complete agglutination.
± Partial agglutination.
— Negative agglutination.

strain of *B. typhosus* in running Widals and to select easily agglutinable strains such as the Mt. Sinai strain (No. 10) or the Rawlings strain (No. 12).

DISCUSSION.

These antigenic differences shown by agglutination, absorption and Widal tests are abundantly confirmed by an examination of the literature. Resistance to agglutination of some strains of *B. typhosus* recently isolated from the blood or feces is well known, and these strains usually become agglutinable, after growth on artificial culture media. We are not concerned with such strains in this case. Sheller (1908) and Burri and Dugeli (1909) report the isolation of organisms from convalescent and healthy carriers culturally identical with *B. typhosus* but which were inagglutinable. Since, at this time, some confusion existed regarding the cultural characteristics of *B. typhosus* their results cannot be accepted without question.

Raubitschek (1913), using organisms isolated from the different organs of a fatal case of typhoid, reports remarkable differences in agglutinability in these organisms which under the conditions of the test would appear to be different individuals of one strain. High-titred serum was prepared by immunizing rabbits with an old laboratory strain. It might be suggested that this strain was one resembling our No. 2 or No. 3 which gave a serum agglutinating few organisms other than the homologous organism. Teague and Torrey (1907) and Taskin, cited by Garbat (1916) report the occurrence of irregular strains of *B. typhosus*. Durham (1901) observed antigenic differences, but did not attempt to group his strains on this basis. Garbat (1916) considered the antigenic differences of enough importance that he used seven or eight strains in the preparation of his antigen for complement-fixation work. Mock (1919) reports atypical serological and cultural reactions in organisms isolated from typhoid patients who had been vaccinated about one year previous to the attack. Myers (1920) reports the isolation of an atypical strain from the blood stream of a vaccinated individual. Hooker (1916) worked out two groups and one subgroup among typhoid organisms by the use of the complement-fixation test. Group I showed cross-fixation with all antigens, group II with each other but not with group I, group I-A gave irregular results. He finds that the Rawlings strain seems to possess less antigenic complexity than does any other organism. It should, therefore, have less value as an immunizing strain. His absorption tests showed that groups I and II

were quite distinct. Weiss (1917) was able to divide his organisms into two well-marked groups, and a third group containing an inagglutinable, rhamnose-fermenting organism. These antigenic differences were correlated with irregularities in xylose fermentation.

It may be seen from Tables III and IV that there exist undoubted antigenic differences in the strains tested. The differences seemed to be quantitative rather than qualitative, since most sera gave agglutination with all organisms in low dilutions.

The antigenic differences became more apparent and seemed to possess more significance in the Widal tests. Table VI indicates the degree to which the organisms differed.

The serological reactions here recorded as evidenced by agglutination, absorption and Widal tests might have an important bearing on the following points:

(1) Sources of error in the Widal run with dried blood.
(2) The occurrence of negative Widals in clinical cases of typhoid fever.
(3) The occurrence of typhoid fever in vaccinated cases.
(4) The advisability of using a polyvalent vaccine.
(5) The correlation between cultural and antigenic differences.

My results indicate that a Widal using dried blood gives a weaker reaction than one run with clear serum. This tends to exaggerate the antigenic differences between the organisms. In Widal tests, an easily agglutinable organism should be chosen, since the less agglutinable types may be entirely negative, even though the same serum gives a complete agglutination with another strain. These results may be due to a decrease in the activity of the agglutinins from drying or to the presence of harmful substances on the material on which the blood is dried.

Stober (1904) and Mock (1919) report irregularities in Widal reactions from clinical cases of typhoid while Robinson (1915), on the other hand, reports no variability in one hundred Widals using the Worcester and Rawlings strains. It is very possible that when using two easily agglutinable strains such as the Rawlings and Mt. Sinai strains no significant differences could be observed. The antigenic peculiarities discussed here might account for the occurrence of negative Widals in typhoid fever. If the strain infecting the patient were not antigenically similar to the strain used for the Widal it is conceivable that an appreciable amount of agglutinin would not be present in the patient's blood for the strain used in the laboratory. Conversely, if the laboratory used one strain only, and

that one happened to be one of the less easily agglutinable strains it is possible that the patient's blood might not agglutinate it. The antigenic differences demonstrated between the Rawlings strain and No. 2 bear out this contention. Hawn, Hopkins and Meader (1918) report forty per cent of positive Widals in a small epidemic among American troops. The source of the organisms used for the Widals is not given. From Table VI it will be seen that the number of positives with Nos. 1, 2 and 3 range from twenty to forty per cent, while with Nos. 12 and 13 they range from seventy to seventy-five per cent. Where the percentage of positive Widals is low, as in Hawn's (1918) cases, the organism infecting the patient or used by the laboratory might be of some importance. It is always possible that a negative Widal may be due to a paratyphoid infection, so that it seems wise to use *B. paratyphosus A* and *B* as well as at least one easily agglutinable strain of *B. typhosus*. Manteufel (1921) would go even farther and set up each Widal with *B. suipestifer* and *B. enteritidis* as well as *B. paratyphosus A* and *B*, and *B typhosus*, basing his recommendation on the occurrence of all these organisms in cases and carriers.

A number of cases of typhoid occurring in vaccinated individuals may be found in the literature. There were numerous cases of "so-called" typhoid reported in the German army and in the French army prior to 1915, when the vaccine containing *B. typhosus, B. paratyphosus A* and *B* was first used. It is reasonable to assume, therefore, that many of these cases were really paratyphoid infections. There were a few undoubted cases of typhoid in the American army among vaccinated soldiers. Brown, Palfrey and Hart (1919) report eighteen cases of clinical typhoid with isolation of the organisms from the blood stream in the majority of cases. These men had been vaccinated within the year with the army vaccine.

Hawn, Hopkins and Meader (1918) report the occurrence of thirty-eight cases of typhoid among vaccinated men, and suggest three possibilities as the cause: First, defective vaccination; second, infection with a different strain; third, overwhelming infection.

Goodall (1918) reports a number of cases among Belgians, French and English. Here, there seems to be some doubt whether the cases were not paratyphoid infections. Protection against paratyphoid was imperfect at this time. Vaughn (1919) says that, "It is possible that in so far as vaccination has failed it is due to the disease being caused by other members of the typhoid group, . . . which in all probability is much larger than we now appreciate." Mock

(1919) reports the occurrence of forty-five cases of typhoid and paratyphoid in individuals who had been vaccinated about one year previous to the attack. Trowbridge (1915) reports the occurrence of a typhoid epidemic among vaccinated persons in an institution, where the original source of infection came from the milk supply which was infected by a vaccinated worker with a mild case of typhoid. It is realized that in such an epidemic the dosage may have been sufficient to overcome the immunity acquired from vaccination. Wade and McDaniels (1915) report the occurrence of an epidemic among vaccinated individuals. Here there seemed to be an interesting correlation between the negative Widals given after vaccination and the susceptibility of these persons to typhoid.

Hooker (1916) and Weiss (1917) conclude from their experiments that a vaccine made from several strains of *B. typhosus* would be more efficient than one made from a single strain. Brown (1919) also suggests this possibility. The results of these observers and the cases of typhoid in vaccinated individuals as cited above, together with my findings would suggest that it might be well to consider the use of a vaccine made from several strains. Teague (1920), on the other hand, rather arbitrarily dismisses the necessity of using more than one strain in vaccines on the grounds that the antigenic differences noted are not permanent. The antigenic differences between my strains have persisted for three years.

An examination of the results reported in experiments I and II showed that there was very little correlation between cultural and antigenic characters. Cultures Nos. 12, 21 and 40, which were irregular in xylose fermentation were all typical in agglutination reactions and belonged to group II, which gave good cross-agglutination with other strains. This is not in agreement with the observations of Weiss (1917). Strain No. 1 gave an unusually rapid reversion to alkaline in litmus milk and was somewhat less agglutinable than many of the other strains. Strains 2 and 3 were typical in their cultural reactions but were less agglutinable than most of my other strains. Strain 5 was inagglutinable and fermented rhamnose but not xylose. It, therefore, differed from the other strains in cultural and antigenic properties. The identity of strain 5 remains unsettled. It gave no agglutination with any of the antityphoid sera used, nor did it reduce the titre in absorption tests. Conversely, the serum produced by the inoculation of this organism into a rabbit did not agglutinate any of the other organisms used, nor did any of the typical typhoid strains reduce the

titre of strain 5 serum. This organism did not agglutinate with stock serum for *B. paratyphosus A* or *B,* nor with a *B. sanguinarium* or *B. pullorum* antiserum. After three years this strain remained inagglutinable in antityphoid serum. Weiss (1917) reported on an entirely inagglutinable strain of this type. Sheller (1908) has reported on an inagglutinable strain. Myers (1920) reported the isolation of a rhamnose-positive *B. typhosus* which was also atypical in its serological reactions. Krumwiede (1918) feels, in view of the apparent serological relations between *B. sanguinarium* and *B. pullorum* and *B. typhosus,* that the fermentation of rhamnose is a more fundamental character than serological reactions. Krumwiede using this fermentation as the deciding factor between the typhoid and paratyphoid group would place the Weiss organism and strain 5 in the paratyphoid group.

CONCLUSIONS.

In summing up the work done the following conclusions were reached:

1. Culturally, the strains of *B. typhosus* used differed slightly from each other, the reaction being most variable in dextrin, xylose, salicin and litmus milk. These variations could not be correlated with the age of the culture nor the source.

2. Cross-agglutination and absorption tests established the existence of quantitative antigenic differences between the strains used. It appeared that the conflicting opinions as to whether there are antigenic differences in the typhoid group may be due to the fact that qualitative rather than quantitative differences have been emphasized.

3. There was no significant correlation between the cultural and antigenic peculiarities demonstrated.

4. The antigenic differences exhibited could not be correlated with the age of the organisms.

5. There was a marked difference in the agglutination of different strains of *B. typhosus* by the serum used in Widals and it would be advisable to set up each Widal with more than one strain, selecting strains which were known to give a high percentage of positive reactions.

6. For the Widal the use of fresh serum drawn from the clot was much more satisfactory than the use of dried blood.

7. An organism in some respects resembling *B. typhosus* but exhibiting cultural and antigenic irregularities was described.

PART II.

METABOLIC STUDIES.

PURPOSE.

The purpose of the second part of my investigations may be summarized as follows:

1. To study the relation of hydrogen-ion concentration to growth, vitality, and fermentation reactions of *B. typhosus*.

2. To study the sugar consumption of *B. typhosus* and correlate this with other factors such as growth rates, titratable acidity and change in hydrogen-ion concentration.

3. To tabulate and compare the characters of all of the strains of *B. typhosus* studied and note any correlation between antigenic variations and other characteristics.

METHODS AND TECHNIQUE.

ELECTROMETRIC. For the measurement of hydrogen-ion concentrations both electrometric and colorimetric methods were used.

The electrometric method was used for checking all buffer solutions and in many of the other determinations where a check on the colorimetric method was desirable or where especial accuracy was required. A Leeds and Northrup potentiometer with a Clark (1922) bubbling electrode was used. In the following tables electrometric determinations may be recognized, as they are expressed in four figures, while no attempt was made to read colorimetric determinations closer than .1 pH.

For the colorimetric determinations buffer solutions, made according to the directions given by Clark and Lubs (1917*a*), were used. These buffer solutions were checked by the electrometric method and corrected when necessary.

The phenol-sulphon-phthalein indicators recommended by Clark and Lubs (1917) were used. A comparator such as the one described by Hurwitz *et al.* (1916) was found satisfactory. The tubes containing the buffer solutions, with indicator added, were superimposed on tubes containing the medium to be tested, diluted 1-4, but without indicator. This compensates for the turbidity and color of the medium to be tested. Clark and Lubs (1917) state that a medium may be diluted four to five times without an appreciable change in hydrogen-ion concentration. This dilution reduces the errors due to turbidity and color. One cubic centimeter of the medium to be tested was diluted to 5 cc. with freshly distilled water

and two drops of indicator added. This tube was placed between the two buffer solutions and the color compared.

Clark and Lubs (1917), Foster (1921 *a* and *b*), Avery and Cullen (1919*a*), Dernby and Avery (1918) and others have reported that accurate readings may be made within 0.05 to 0.1 pH if the proper precautions are observed. This also has been my experience.

CULTURE MEDIA.

For viability and purity tests eosin-methylene-blue lactose agar plates, Holt-Harris and Teague (1916) were used. For the determination of growth rates, meat-infusion broth agar adjusted to pH 7.2 was found to be satisfactory. For the metabolism work meat-infusion broth was used, to which was added buffer solutions as indicated in the experiments and the percentage of carbohydrates desired.

EXPERIMENTAL.

The studies included in the following experimental work comprise a study of:

1. Effect of hydrogen-ion concentration on *B. typhosus*.
   (*a*) The range of growth of *B. typhosus*.
   (*b*) The change of hydrogen-ion concentration in plain broth.
   (*c*) The final or limiting hydrogen-ion concentration in broth containing various fermentable carbohydrates.
   (*d*) The viability of *B. typhosus* in broth containing a fermentable carbohydrate.
   (*e*) The influence of varying factors on the final hydrogen-ion concentration in dextrose broth.
2. Dextrose metabolism and correlated factors.
   (*a*) Utilization of glucose and influence of buffers.
   (*b*) Rate of acid formation.
   (*c*) Growth rates.
   (*d*) Rate and relation of the utilization of dextrose to final hydrogen-ion concentration and titratable acidity, inhibition and death.

QUALITATIVE TESTS.—EFFECT OF HYDROGEN-ION CONCENTRATION ON B. TYPHOSUS.

(*a*) *Range of Growth for B. typhosus.*

MEDIA. Meat infusion broth was titrated, using phenolphthalein, and divided into nine lots of 150 cc. each in hard-glass flasks. A calculated amount of acid, N/1 HCl, and alkali, N/1 NaOH, as the case might be, was added to bring the reaction to the approximate hydrogen-ion concentration desired. Each lot was then tested colorimetrically with a suitable indicator and further adjustment made

Table VII.—Range of growth of *B. typhosus* in plain broth expressed in logs.

Organism.	Original number of organisms.	pH 4.564.	pH 5.001.	pH 5.764.	pH 6.576.	pH 7.434.	pH 7.658.	pH 8.115.	pH 8.402.	pH 9.636.
12	5.9212	sterile	4.5315	8.8949	9.0000	9.0086	8.6021	7.8388	7.3324	7.3617
5	6.5717	sterile	sterile	7.7212	9.0569	8.9345	9.1461	9.1461	8.8865	8.3010
1	4.9542	sterile	sterile	7.4771	7.9085	8.6021	8.2041	8.3010	6.7782	sterile
2	5.6180	sterile	4.5315	7.9213	7.7243	8.8513	8.7782	7.8865	6.4683	sterile
9	5.8651	sterile	sterile	8.6857	8.9058	9.0086	8.0453	7.8386	7.3321	sterile
4	5.7924	sterile	sterile	7.4771	7.9085	7.9542	8.2041	7.9581	6.7782	sterile
13	5.7709	sterile	sterile	7.9959	7.9325	8.9494	8.8808	7.8195	6.4624	sterile

if necessary. The media was then sterilized and electrometric determination made just before inoculation. Each flask was inoculated with 0.5 cc. of an eighteen-hour broth culture. A number of dilutions in sterile water blanks were made immediately and plated. The average number of colonies from all the flasks was taken as shown in Table VII. It was found necessary to make the plates immediately, since a decrease of organisms was observed in the more acid flasks shortly after inoculation. After eighteen hours in the incubator 5 cc. were removed from each flask, the proper dilutions made and plated. All plates were made in duplicate or triplicate. A sample was also removed for hydrogen-ion determination. In all cases where growth had occurred there was a slight increase in alkalinity from 0.1 to 0.3 pH.

Table VII shows the range of growth of organisms which represent typical examples taken from many determinations. It is evident in every case that no growth was present after eighteen hours at pH 4.5, that is to say, this degree of acid had sterilized the medium. At pH 5.0 only two organisms, No. 12 and No. 2, were viable, and the number of organisms was less than the original. The maximum growth took place between pH 6.0 and pH 7.5 with the exception of No. 5, where growth was vigorous, but decreasing at pH 9.6. With the exception of Nos. 12 and 5 all were sterile at pH 9.6.

Fennel and Fisher (1919) found that *B. typhosus* grew on nutrient agar from pH 4.0 to pH 9.6 with an optimum of pH 6.2 to pH 7.2. Dernby (1921) gives the optimum as pH 6.8 to 7.2. Schoenholz and Meyer (1921), using a heavily buffered meat infusion broth, found that growth was slight or none at pH 4.3 and pH 4.8; slight at pH 9.0, and most vigorous at pH 6.6 and pH 6.8. They also make the statement that although *B. typhosus* grows better in a slightly acid environment, it is more tolerant of alkali than of acid. This was found to be the case in my experiments also. These same authors found that in a medium containing bile salts growth was greatly inhibited at pH 8.2. Wright (1917) observed that the growth of *B. typhosus* was light at pH 4.5-5.0, heaviest at pH 6.0-6.5 and declined sharply at pH 7.5-8.0. Dernby and Näslund (1923) give the limits for growth for *B. typhosus* as pH 4.6-8.0 with an optimum of pH 6.5-7.2. Hall and Fraser (1922) give the limits of growth at pH 4.0-8.6, using a peptone solution made from pure casein.

My results are comparable to those of Schoenholz and Meyer (1921), Wright (1917), Dernby and Näslund (1923), and Hall

and Fraser (1922). Fennel and Fisher's (1919) results suggest either that the organisms grew over a wider range of hydrogen-ion concentration in solid media than in liquid media, or that these authors were using an organism similar to No. 12 or No. 5, which exhibited unusually vigorous growth over a wide range. With the exception of strains 12, 2 and 5 the limits of growth for my organisms were pH 5.5-8.5. The optimum was from pH 6.0 to 7.5 and the different strains did not differ markedly from one another. The limits of growth for No. 2 was from pH 5.0 to pH 8.5, for No. 12 from pH 5.0 to pH 9.0, and No. 5 from pH 5.5 to pH 9.6. It is interesting to observe that antigenically these organisms were quite distinct, No. 2 belonging to group III and interagglutinating well only with No. 3. No. 12, the Rawlings strain, is a member of the larger and more homogeneous group II, and No. 5 is the inagglutinable strain.

From my results and those of the authors cited, it is evident that *B. typhosus* is unable to withstand pH 4.5 for twenty-four hours, and is able to multiply feebly at pH 5.0 on the acid side. It multiplies fairly well at pH 8.0, but from that point on growth is increasingly inhibited, until at pH 9.0 the great majority of strains are sterile after twenty-four hours.

The optimum range is wider in my series than in that of most authors with the exception of Dernby and Näslund (1923). However, the end point as well as the optimum zone depends upon the amount inoculated, the age of the inoculum and the composition of the medium. There was no apparent relation between the length of time the cultures had been isolated and the optimum zone, the most recently isolated culture showing no marked difference from those which had been growing on culture media for thirty-three years. This is not in accord with the findings of Schoenholz and Meyer (1921). Such a range of optimum growth gives a fairly wide latitude in the selection and adjustment of culture media. This is in marked contrast to an organism like the pneumococcus where the range of optimum growth occurs between pH 7.4-7.8.

It may be pointed out, however, that the reaction of Endo's medium, pH 7.8-8.4, would inhibit the growth of *B. typhosus* to a certain extent.

(*b*) *Change of Hydrogen-ion Concentration in Plain Broth.*

It is difficult to understand why the statement is so frequently made that the growth of organisms in plain broth produces no change in reaction. Russel *et al.*, cited by Esty (1921), and Dernby (1923), state that there is no change in the reaction of culture media produced by *B. typhosus* if the medium is free of dextrose. Hajes (1922) states that there is no change in hydrogen-ion concentration in plain broth medium using a variety of organisms. D'Aunoy (1923), using *B. pestis* and the colorimetric method, found no change in hydrogen-ion concentration in plain broth medium. In contrast to the above statements, are those of Peré (1892), Hulton-Fraenkel (1919*a*), and others, that an increased alkalinity is present after several day's incubation in plain broth or in broth containing so small an amount of carbohydrate that it may be completely used. Cobbett (1897), Bunker (1919), Hartley (1922), and others, have shown that a distinct alkaline reversion takes place in diphtheria cultures in a medium containing a small amount of dextrose, and that this alkaline reversion is synchronous with toxin production.

Table VIII gives a summary of the titratable acidity and change in hydrogen-ion concentration in a highly buffered broth produced by twenty-five typical strains of *B. typhosus* and one atypical strain, No. 5. It will be seen that there was a slight increase in titratable acid during the first twenty-four hours, accompanied by a small increase in hydrogen-ions which was within the limits of error for the colorimetric test. In forty-eight and seventy-two hours there was a progressive decrease in titratable acidity and decrease in free hydrogen-ions.

With the exception of No. 5, the changes in hydrogen-ion concentration in plain meat infusion broth were remarkably constant for the different strains. Strain 5 showed a more marked alkaline production than did the others. This strain also differed antigenically. This progressive alkalinity was observed in all of our cultures under the following conditions: First, meat-infusion broth to which no fermentable carbohydrate was added; second, broth to which such small amounts of carbohydrate was added that the organisms were able to ferment it completely without marked inhibition of growth; third, broth to which a carbohydrate was added which was not attacked by *B. typhosus*, such as saccharose.

The slight increase in titratable acidity was probably due to the fermentation of the small amount of dextrose present in meat in-

fusion broth. Kendall (1913) suggests that this increased acid might also be due to the fermentation of the so-called carbohydrate moiety of some of the proteins in the peptone. He attributes the

TABLE VIII.—Titratable acidity and hydrogen-ion concentration in buffered meat-infusion broth to which no dextrose was added. Summary of results with twenty-five organisms and strain No. 5 listed separately.

ORGANISM.	Original pH.	Titratable acidity.	pH 24 hours.	Titratable acidity 24 hours.	pH 48 hours.	Titratable acidity 48 hours.	pH 72 hours.	Titratable acidity 72 hours.
Summary.....	7.8	4.47	7.7	4.95	8.03	4.18	8.15	2.42
No. 5.....	7.8	4.48	7.6	6.02	8.2	4.80	8.6	1.42

progressive alkalinity to an increase in alkaline products, chiefly ammonia from the proteins. The organisms deamidize the proteins prior to the intracellular use of the nitrogen-free portion for energy.

It seems likely that those persons who did not observe this alkalinity failed to do so because of the lack of suitable indicators or the failure to make observations over a sufficient period of time.

(c) *The Final Hydrogen-ion Concentration in Broth Containing Various Fermentable Carbohydrates.*

This experiment may be divided into three parts: First, the final or limiting hydrogen-ion concentration in 1.0 per cent carbohydrate broth; second, the final hydrogen-ion concentration in 2.0 per cent carbohydrate broth; third, the effect on fermentation of growth in the presence of a carbohydrate which is fermented with difficulty.

In every case the desired amount of sterile carbohydrate was added to sterile meat-infusion broth adjusted to the hydrogen-ion concentration indicated in the tables. The hydrogen-ion concentration was determined in some cases by electrometric, in others by colorimetric methods and the results were checked many times. Dextrose, mannite, maltose, xylose and dextrin were used, as it was felt that these carbohydrates were representative of the different carbohydrates fermented by *B. typhosus*.

TABLE IX.—Hydrogen-ion concentration and day of death in 1 per cent dextrose broth.

ORGANISM.	Original pH.	pH 24 hours.	pH 48 hours.	pH 72 hours.	Growth, 72 hours.
1	7.0	5.1	5.2	5.2	0
2	7.2	5.1	5.2	5.2	0
3		5.1	5.1	5.0	0
4	7.0	5.0	4.9	5.2	+
5	7.0	5.2	5.5	5.5	+
9	7.0	5.1	5.1	5.1	0
12	7.0	5.2	5.2	5.2	0
13	7.0	5.2	5.2	5.1	0
14	7.1	5.0	5.0	5.1	0
15	7.2	4.9	5.0	5.0	0
17	7.1	4.9	5.1	5.2	0
21	7.2	4.9	4.9	4.9	+
23	7.1	5.0	4.9	4.9	+
24	7.2	5.0	5.1	5.0	0
25	7.0	5.0	5.1	5.2	0
26	7.0	4.9	5.0	5.1	0
27	7.0	5.1	5.1	5.2	0
28	7.0	5.1	5.1	5.1	+
29	7.1	5.1	5.1	5.0	0
30	7.2	5.1	5.1	5.1	0
32	6.8	5.0	5.0	5.1	+
39	6.8	5.1	5.1	5.2	0
40	7.2	5.0	5.0	5.1	0
44	6.8	5.1	5.1	5.2	0
45		5.1	5.1	5.1	0

Table IX gives the hydrogen-ion concentration in 1 per cent dextrose broth. It will be seen that in every case the final hydrogen-ion concentration was reached in twenty-four hours. The final hydrogen-ion concentration reached in most cases was pH 5.1 and the greatest was pH 4.9. In a few cases there was a slight alkaline reversion after seventy-two hours, or one week, amounting to 0.1-0.3 pH. Six organisms showed growth after seventy-two hours, and one only, No. 5, after one week.

TABLE X.—Hydrogen-ion concentration and day of death in 1 per cent mannite broth.

ORGANISMS.	Original pH.	pH 24 hours.	pH 48 hours.	pH 72 hours.	Growth, 72 hours.
1	7.2	5.3	5.3	5.4	0
2	7.2	5.2	5.3	5.4	0
3	7.2	5.2	5.2	5.3	+
4	7.2	5.2	5.2	5.3	+
5	7.2	5.6	5.8	5.8	0
9	7.2	5.3	5.3	5.3	0
12	7.4	5.1	5.0	5.2	0
13	7.4	5.1	5.2	5.5	+
14	7.4	5.2	5.2	5.3	+
17	7.4	5.2	5.1	5.1	0
21	7.4	5.1	5.1	5.3	0
23	7.4	5.3	5.1	5.2	0
24	7.4	5.2	5.1	5.4	0
25	7.4	5.2	5.2	5.0	+
26	7.4	5.2	5.3	5.3	0
27	7.4	5.1	5.1	5.3	0
28	7.4	5.1	5.2	5.5	0
29	7.4	5.1	5.1	5.3	0
30	7.4	5.1	5.2	5.4	0
32	7.4	5.2	5.2	5.4	0
39	7.4	5.1	5.1	5.2	+
40	7.4	5.2	5.1	5.2	0
44	7.4	5.1	5.2	5.3	0
45	7.4	5.1	5.1	5.1	0

Table X gives the hydrogen-ion concentration in 1 per cent mannite broth. The majority of strains reached a pH of 5.1 but none were more acid than this. With four exceptions, this final hydrogen-ion concentration was reached in twenty-four hours. All showed slight alkaline reversion after one week. Six out of twenty-five strains were not viable in seventy-two hours.

TABLE XI.—Hydrogen-ion concentration and day of death in 1 per cent maltose broth.

ORGANISM.	Original pH.	pH 24 hours.	pH 48 hours.	pH 72 hours.	Growth, 72 hours.	1 Week.	Growth, 2 weeks.
1	7.6	6.021	6.0	6.0	+ +	5.8	+ +
2	7.6	6.2	5.7	5.4	+ +	5.7	— —
3	7.6	6.021	5.528	5.782	+ +	5.444	+ +
4	7.6	5.6	6.0			6.0	— —
5	7.6	6.195	5.985	8.098	+ +	8.706	+ +
9	7.6	5.8	5.5	5.6	+ +	5.8	— —
12	7.6	6.021	5.545	5.579	+ +	5.511	— —
13	7.6	6.1	5.8	5.4	+ +	5.8	+ +
14	7.6	6.1	6.2	5.6	+ +	5.6	— —
17	7.6	6.8	6.2	5.6	+ +	5.6	— —
21	7.6	6.096	5.579	5.647	+ +	5.444	— —
23	7.6	6.1	6.1	5.4	+ +	5.7	+ +
24	7.6	6.1	6.1	5.3	+ +	5.8	+ +
25	7.6	6.1	5.7	5.7	+ +	5.7	+ +
26	7.6	6.1	5.6	5.4	+ +	5.8	—
27	7.6	6.2	5.8	5.4	+ +	5.8	+ +
28	7.6	6.3	5.5	5.4	+ +	6.4	+ +
29	7.6	6.2	5.8	5.4	+ +	5.8	+ +
30	7.6	6.1	5.7	5.5	+ +	5.8	— —
32	7.6	6.1	5.6	5.4	+ +	5.8	+ +
39	7.6	6.2	5.5	5.5	+ +	5.6	+ +
40	7.6	6.3	5.4	5.6	+ +	5.6	— —
44	7.6	5.9	5.8	5.7	+ +	5.8	—
45	7.6	5.9	6.0	5.5	+ +	5.8	—

Table XI gives the hydrogen-ion concentration in maltose broth. There is much greater variation in the final hydrogen-ion concentration reached and the length of time involved. The final hydrogen-ion concentration reached was from pH 5.3 to pH 6.1. It will be observed that most of the organisms reached their final hydrogen-ion concentration only after seventy-two hours incubation. The highest hydrogen-ion concentration reached in twenty-four hours was pH 5.6. After seventy-two hours' incubation, all organisms were living. After one week, twelve were living and thirteen were dead. Strain No. 5 differed from the other organisms in showing marked alkaline reversion after forty-eight hours. This was progressive in spite of the continued presence of maltose in the medium.

TABLE XII.—Hydrogen-ion concentration and day of death in 1 per cent xylose broth.

ORGANISM.	Original pH.	pH 24 hours.	pH 48 hours.	pH 72 hours.	Growth, 72 hours.	pH 1 week.	Growth, 1 week.
1	7.2	5.6	5.6	5.7	+	6.2	—
2	6.948	5.292	5.258	5.172	—	5.258	—
3	6.9	5.6	5.6	5.7	+	5.9	—
4	6.9	5.6	5.4	5.8	+	5.8	—
5	6.931	7.303	7.777	8.284	+	8.537	+
9	7.2	5.4	5.5	5.6	+	5.9	+
12	7.236	7.151	7.523	7.574	+	6.728	+ +
13	7.185	5.5	5.5	6.0	+	6.5	+ +
14	7.1	5.5	5.5	5.6	+	5.6	+
15	7.0	5.5	5.5	5.6	+	5.9	+
17	7.2	5.4	5.4	6.0	+	6.2	—
21	6.931	7.320	7.608	7.743	+	7.658	+
23	7.2	5.8	5.8	6.0	+	6.2	+
24	7.2	5.4	5.4	5.7	+	5.7	+
25	7.1	5.5	5.5	5.8	+	5.8	+
26	7.2	5.6	5.6	5.6	+	5.7	+
27	7.1	5.4	5.4	5.8	+	5.8	—
28	7.1	5.4	5.4	5.6	+	5.6	—
29	6.914	5.376	5.258	5.292	—	5.697	—
30	6.914	5.241	5.292	5.292	—	5.711	—
32	7.2	5.4	5.4	5.6	+	5.7	—
34	6.9	5.5	5.6	5.6	+	5.6	—
40	6.948	6.661	6.948	7.067	+	7.236	+
44	6.948	5.3	5.3	5.7	+	5.9	+
45	6.9	6.2	6.0	6.0	+	6.6	+

Table XII gives the final hydrogen-ion concentration and the day of death of *B. typhosus* in 1 per cent xylose broth. The lowest point, pH 5.1, was reached by only one organism and after seventy-two hours' incubation. The hydrogen-ion concentration reached by the majority was from pH 5.2 to 5.6. There was slight alkaline reversion in all tests, and all organisms were viable after seventy-two hours. Eleven organisms were not viable after one week. Three organisms, namely: Nos. 5, 21 and 40, did not ferment xylose at all during the period covered in the table. The broth became progressively alkalin as if there were no carbohydrate present. The Rawlings strain, No. 12, gave slight acid after one week. No. 21 produced slight acid

in twenty-four hours and then became progressively alkaline for about two weeks. At the end of three weeks it had reached pH 6.5. At the end of three weeks No. 12 and No. 40 had reached pH 5.5 and 5.8, respectively. Strain No. 5 produced a progressively alkaline reaction, giving pH 8.4 at the end of three weeks.

TABLE XIII.—Hydrogen-ion concentration and day of death in 1 per cent dextrin broth.

ORGANISM.	Original pH.	pH 24 hours.	pH 48 hours.	pH 72 hours.	Growth, 72 hours.	pH 1 week.	Growth, 1 week.
1	7.2	6.2	7.0	7.4	+	7.5	+
2	7.2	6.2	7.0	7.0	+	7.6	+
3	7.2	5.6	6.4	6.6	+	7.5	+
4	7.2	6.6	6.8	6.8	+	7.2	+
5	7.2	5.6	7.2	7.2	+	7.5	+
9	7.2	5.6	5.5	6.0	+	7.5	+
12	7.2	6.2	6.8	6.8	+	7.6	+
13	7.2	6.4	6.8	6.8	+	7.5	+
14	7.2	6.3	6.0	6.9	+	7.4	+
15	7.2	5.6	6.3	6.9	+	7.4	+
17	7.2	5.2	6.1	6.7	+	7.3	+
21	7.2	5.9	6.3	7.0	+	7.3	+
23	7.2	6.0	6.5	6.8	+	7.2	+
24	7.2	5.9	5.8	6.8	+	7.4	+
25	7.2	6.4	7.3	7.3	+	7.6	+
26	7.2	6.2	6.8	6.8	+	7.8	+
27	7.2	5.8	5.8	5.8	+	7.6	+
28	7.2	5.9	6.8	6.9	+	7.4	+
29	7.2	5.9	6.4	6.7	+	7.3	+
30	7.2	5.9	5.8	6.8	+	7.4	+
39	7.2	5.9	6.8	6.9	+	7.4	+
40	7.2	5.9	6.8	6.8	+	7.6	+
44	7.2	6.0	6.6	6.8	+	7.8	+
45	7.2	6.1	6.7	6.9	+	7.8	+

Table XIII gives the results in dextrin broth. These determinations were unsatisfactory because of the insolubility of the dextrin. Although several samples were tried, none were entirely satisfactory. *B. typhosus* seemed to attack this carbohydrate with difficulty. A slight transient acidity was produced in all cases with an alkaline reversion, beginning after forty-eight hours and continuing during the period of incubation. After one week the hydrogen-ion con-

Table XIV.—Summary of final hydrogen-ion concentration in meat-infusion broth containing 2 per cent of the fermentable carbohydrates.

Organism.	2 per cent dextrose.	Growth, 72 hours.	2 per cent mannite.	72 hours.	27. Maltose.	72 hours.	27. Xylose.	72 hours.	27. Dextrin.	72 hours.
Average, 31 organisms	pH 4.9–5.1	0	pH 5.0–5.2	0	pH 5.1–5.6	+0	pH 4.7–5.2, except 21, 40 and 12	0	pH 5.6–6.6	+
No. 5	pH 5.1	+	pH 5.0	+	pH 5.2	+	alkaline	+	pH 6.6	+

+ No acid in seventy-two hours.

centration averaged 0.1-0.6 pH higher than the original reaction. All the organisms were viable.

No constant differences could be observed in the final hydrogen-ion concentration reached by the different strains and there were no differences which could be correlated with antigenic differences in dextrose, mannite, maltose and dextrin broth. The results in xylose broth are discussed separately.

Table XIV gives a summary of the results obtained using 2 per cent carbohydrate broth. In dextrose, the final hydrogen-ion concentration was practically the same as in 1 per cent broth. In mannite, maltose and xylose the hydrogen-ion concentration was slightly greater in 2 per cent than in 1 per cent broth. It was hard to account for this, since it was usually found that 1 per cent of the carbohydrate was not completely used. My later studies show that few of the organisms were able to use 1 per cent dextrose. Fred and Peterson (1920) found that *B. typhosus* gave the maximum acid yield in 2 per cent xylose although only 25 per cent of the sugar was used. In 2 per cent dextrin the hydrogen-ion concentration did not differ from that in 1 per cent broth.

TABLE XV.—Hydrogen-ion concentration in 2 per cent xylose broth.

ORGANISM.	Original 2 pH.	24 hours.	48 hours.	72 hours.	7 days.	14 days.	21 days.
12	6.914	6.864	7.472	7.774	6.340	5.714	5.528
5	6.914	6.948	7.760	8.132	8.487	8.470	8.419
21	6.914	6.999	7.591	7.506	6.982	6.779	6.627
40	6.948	6.661	6.948	7.067	7.236	6.593	5.866

Table XV gives the result of the hydrogen-ion concentration determinations in 2 per cent xylose broth of Nos. 5, 12, 21 and 40 after repeated transfers in xylose broth. It was hoped that the organisms might be trained to ferment xylose more rapidly. This was not accomplished.

(d) Viability in Broth Containing a Fermentable Carbohydrate.

With very few exceptions growth was tested for at seventy-two hours and thereafter whenever a hydrogen-ion determination was made. Summarizing our results, we find that when pH 5.0 to pH 5.2 was reached the organisms were rarely viable after seventy-two hours. If pH 5.4 to pH 5.5 was reached, the organisms were usually viable after one week, but many were not viable by the end of the

second week. When a lower hydrogen-ion concentration than pH 5.5 was reached, the majority were viable at the end of the third week. The organisms in 1 per cent mannite and maltose broth lived longer than in dextrose broth. This agrees with the work of Ishii (1920). The greater viability in carbohydrates other than dextrose is probably due to the higher hydrogen-ion concentration reached in dextrose broth. In 1 per cent xylose and dextrin broth where the hydrogen-ion concentration was lower than in dextrose, mannite or maltose, the organisms lived much longer. In 2 per cent mannite, maltose and xylose broth, where a greater concentration of hydrogen-ions was reached, the organisms were only slightly more viable than in dextrose broth.

The viability of the organisms could not be correlated with any antigenic differences noted previously except in the case of No. 5, which lived longer than the other strains. This viability is associated with the lower degree of hydrogen-ion concentration produced in most carbohydrates, with the more vigorous alkalin production and greater growth range.

(e) *The Influence of Varying Factors on the Final Hydrogen-ion Concentration in Dextrose Broth.*

Influence of varying amounts of glucose and the addition of buffers to the medium. Flasks of meat-infusion broth to which was added the percentage of dextrose desired were inoculated with .1 cc. of an eighteen-hour, second-generation broth culture of the different strains of *B. typhosus*. The reaction in twenty-four hours was tested by the colorimetric method. Four-tenths to six-tenths percent of dextrose was required to give the characteristic limiting hydrogen-ion concentration in meat infusion broth and from 0.6-0.8 per cent in meat-infusion broth to which dipotassium hydrogen phosphate had been added to act as a buffer. The final hydrogen-ion concentration was seen to be the same, although it is obvious that more acid must have been produced in the buffered than in the unbuffered broth. No significant differences between the strains could be found.

DISCUSSION.

The final hydrogen-ion concentration may be defined as the degree of acid produced by an organism in a utilizable carbohydrate. Jones, F. S. (1920), Jones, H. M. (1920), Dernby and Näslund (1923), called attention to the necessity for defining the conditions under which this final hydrogen-ion concentration is obtained. The

initial acidity, the kind and amount of carbohydrate, the age and amount of inoculum and the buffer effect of the medium all affect the limiting hydrogen-ion concentration. Levine (1917) points out that the final hydrogen-ion concentration represents not only the amount of acid formed from the carbohydrate fermented, but that it represents the sum of the metabolic activities of the organisms under the conditions stated.

The questions involved in this discussion are as follows: First, the effect of the carbohydrate on the final hydrogen-ion concentration attained; second, the constancy of the final hydrogen-ion concentration in any one carbohydrate; third, the amount of dextrose needed to produce the final hydrogen-ion concentration and the influence of buffers; fourth, the acid death point and its relation to the final hydrogen-ion concentration; fifth, the alkaline reversion observed after the final hydrogen-ion concentration is reached; sixth, the variations in the above reactions between the individual strains and the relation of such variations to antigenic differences.

FINAL HYDROGEN-ION CONCENTRATION IN DIFFERENT SUGARS.

The sugars used by the author were selected because they represent those most commonly used for the identification of *B. typhosus* and should be representative of the fundamental functional activity of this organism. Dextrose and maltose are always fermented by *B. typhosus;* mannitol is usually fermented. Hulton-Fraenkel (1919 *b*) reports that no acid formation took place in her synthetic medium to which mannite was added. I would suggest that this might have been due to slight growth. Ecker (1922) reports the occurrence of a serologically atypical strain of *B. typhosus* which did not produce acid in mannite. Xylose is usually fermented by *B. typhosus.*

There are many interesting observations in the literature as to the amount of acid produced by any one organism in different fermentable carbohydrates. It would seem that if acidity is the functional ability of the organism to resist acid, the same degree of acidity should be reached in any fermentable carbohydrate. That this may not be true may be seen from the following review and from my own results.

Broadhurst (1913), Smillie (1917), Jones (1918 *a* and *b*), Thro (1915) and Ayres (1918) record a variation in the final hydrogen-ion concentration reached by streptococci in different fermentable carbohydrates. Oppenheim (1920) records the greatest variation in

acid fermentation from streptococci in mannite broth. Julianelle (1922), using hemolytic staphylococci, observed variation in acid production in various carbohydrates. Bengston (1919) studying *B. vulgaris*, records the greatest acidity from the fermentation of dextrose. Fisher (1918) and Michaelis and Marcora (1912) observed that *B. coli* produced greater acidity in dextrose than in lactose broth. Browne (1914) using *B. coli*, correlates the amount of acid produced with the complexity of the sugar fermented. He suggests that the lower acidity in complex sugars is the result of some other decomposition product which accompanies the acids, and in connection with them is able to limit growth. Hall and Randall (1922) report the same acid limits in any fermentable carbohydrate using *B. welchii*. Avery and Cullen (1919 *a* and *b*), Ayers and Mudge (1922), have maintained that the final reaction is the same whenever the carbohydrate is utilizable at all and the conditions of fermentation are comparable. Comparative studies of the fermentation of many strains of organisms in different carbohydrates have not been made except in the case of the streptococci and *B. coli*. As a rule the differences in fermentation between different strains of the same organisms have not been significant.

The final hydrogen-ion concentration reached by *B. typhosus* in dextrose broth is given in the literature as follows: Kendall (1923), pH 5.0; Yuri (1923), pH 4.7 to 5.0; Hulton-Fraenkel (1919*b*), pH 6.5; Kligler (1916), pH 5.0 to 4.8 in peptone water, buffered, pH 4.6 to 4.8 in peptone water, without buffer; Winslow, Kligler and Rothburg (1919), pH 4.9. The last mentioned authors give the final hydrogen-ion concentration in mannite broth as pH 5.1. The final hydrogen-ion concentration in other carbohydrate broths is not recorded.

My results agreed with most of the investigators quoted above in that a distinct difference in the final hydrogen-ion concentration was evident in the different carbohydrates. Dextrose and mannite appeared to be most available judging from the amount of acid produced. Maltose, xylose and dextrin were less available. Practically all investigators agree that the concentration of hydrogen-ions is higher in dextrose than in other carbohydrates. My results were entirely in agreement with this. The use of two per cent carbohydrate tended to lessen the difference in the amount of acid produced in dextrose, mannite and maltose, but not in xylose and dextrin. The utilization of xylose by the so-called slow-fermenters merits special comment. From thirty-two cultures three of my

strains fermented xylose slowly, and one, No. 5, was entirely unable to ferment xylose. This is probably an unusually large percentage of slow fermenters.

Teague (1920) and Morishima (1921) record that out of one hundred and fifteen strains of *B. typhosus* three only remained negative in xylose broth on the twenty-seventh day. When transferred repeatedly in xylose broth two strains became positive, one only remaining negative. All strains on plating gave daughter colonies and by picking colonies showing acid on the xylose plates rapid fermenters might be selected. My results so far have failed to confirm those of the above authors. No. 5 has continued completely negative in xylose broth for thirty days after inoculation. It reached the same degree of alkalinity as if it were in plain broth. Nos. 12, 21 and 40 gave slight acid after one to twenty-one days. None of these cultures have been trained to ferment xylose rapidly by repeated transfers. They continued to be viable as long as if they were in plain broth.

My results suggest that the lower acidity in carbohydrate broth other than dextrose was not due to inhibition of growth, but rather that there was inability to make further use of the carbohydrate. The possibility that an equilibrium was reached in which basic products balance acid formation must be considered also. Levine (1916) points out that the final hydrogen-ion concentration is first, a measure of resistance to acid in some cases as with *B. coli* in dextrose broth, or second, the point of equilibrium between the decomposing carbohydrate and its end products, as in *B. aerogenes* cultures in dextrose broth.

With the exception of No. 5 no constant nor significant differences in the final hydrogen-ion concentration were observed between the different strains in dextrose, mannite, maltose and dextrin. No correlation between antigenic differences and the hydrogen-ion concentration reached by the different strains could be found.

The organisms which fermented xylose easily likewise showed no significant variations. The slow fermenters, Nos. 21, 40 and 12, belonged to the same antigenic group and were antigenically related to the majority of the strains, although they differed from them in the rate at which xylose was fermented.

I am in agreement with Teague (1920), who feels that whenever an organism decomposes a carbohydrate it should be called a positive fermentation test regardless of whether the acid appears in twenty-four hours or after three weeks. Any fermentation test

should extend over a sufficient length of time so the strains which ferment the carbohydrate slowly would be found. In any negative fermentation test there is the possibility that simultaneous alkaline production may mask acid production. Quantitative sugar determinations obviate this difficulty.

CONSTANCY OF HYDROGEN-ION CONCENTRATION IN ANY ONE CARBOHYDRATE.

It is conceivable that all organisms in one group might be capable of producing a constant and characteristic hydrogen-ion concentration in any one carbohydrate under stated conditions. Jones, H. M. (1920), remarks on the constancy of the limiting hydrogen-ion concentration for streptococci, and also for *B. typhosus*, under stated conditions. Avery and Cullen (1919), using pneumococci, observed similar constancy in the limiting hydrogen-ion concentration for this group. The limiting hydrogen-ion concentration in dextrose broth has been considered sufficiently constant under certain conditions to form a method of distinguishing between streptococci of bovine and human origin. Avery and Cullen (1919), Jones (1920), Brown (1920), Ayers and Rupp (1922), and Fuller and Armstrong (1913), have observed constant differences between bovine and human strains of streptococci. Ayers, Johnson and Davis (1918) have proposed using the final hydrogen-ion concentration as a basis for distinguishing between pathogenic and nonpathogenic strains of streptococci. Clark and Lubs (1915) found the hydrogen-ion concentration of *B. coli* in dextrose broth sufficiently constant to make it the basis of their methyl-red test. Cole and Onslow (1916) suggest the use of phenol red to distinguish between *B. paratyphosus A* and *B* and *B. typhosus*. They found that when two-tenths per cent glucose in a casein digest broth is used, in twenty-four hours *B. typhosus* and *B. paratyphosus A* gave a yellow or acid reaction, while *B. paratyphosus B* gave an alkaline or red color. From my results on buffers, it will be seen that such a reaction would hold true only for a medium in which the buffer content was slight. In a well-buffered medium *B. typhosus* might produce an alkaline reaction also in twenty-four hours if only two-tenths per cent of dextrose were present. Koser (1921) shows that in forty-eight hours animal strains of *B. paratyphosus* give pH 5.0 to 5.2, human strains give pH 5.6 to 5.8.

In a large series of experiments I found that the limiting hydrogen-ion concentration as reached by *B. typhosus* was remarkably

constant for each carbohydrate used provided that the amount of inoculum, the composition of the medium and the initial reaction in the different experiments were alike. There were no marked differences in hydrogen-ion concentration between the different strains in any one carbohydrate, with the exception of No. 5, which showed alkaline reversion in seventy-two hours. It was found possible to repeat these experiments many times, obtaining the same range of hydrogen-ion concentration for the different strains each time, providing the other factors were constant.

Yuri (1923) reports a pH of 4.7 to 5.0 for *B. typhosus* in dextrose meat-infusion broth. I found that only occasionally did any of my strains give a hydrogen-ion concentration as high as pH 4.7. Kendall (1921) reports that his strains of *B. typhosus* reached pH 5.0. This agrees with my results. A comparison of *B. typhosus* with other organisms shows that bovine streptococci have a limiting hydrogen-ion concentration in dextrose of pH 4.5 to 4.9; human streptococci pH 5.1 to 5.5; pneumococci pH 5.0; that is to say, these cocci give a higher concentration of hydrogen-ions in the case of bovine streptococci than *B. typhosus* and the human strains about the same as *B. typhosus*. Sherwood* found that a large series of *B. enteritidis* organisms from influenza cases gave a final hydrogen-ion concentration of pH 4.7 to 5.0. Others have reported that the paratyphoid group had a final hydrogen-ion concentration of pH 4.6 to 4.9; that is, slightly more acid than *B. typhosus*. Yuri (1923) reports several strains of *B. dysenteriæ* as producing a final hydrogen-ion concentration of pH 5.2 to 5.6 in dextrose meat infusion broth. This is slightly less acid than most of my strains of *B. typhosus*. The figures for *B. coli* are given from pH 4.5 to 4.8 in dextrose and from pH 4.8 to 5.0 in lactose. The final hydrogen-ion concentration in lactose is more nearly comparable then, to that of *B. typhosus* than it is in dextrose. It may be seen from the foregoing that the final hydrogen-ion concentration of organisms of different or related species is very similar in dextrose broth. Figures for the final hydrogen-ion concentration produced by various species in other carbohydrates which are fermentable by *B. typhosus* are not available for comparison.

I found that the final hydrogen-ion concentration of my different strains was quite constant in each of the different carbohydrates used with the possible exception of dextrin and xylose. In dextrose the final hydrogen-ion concentration was from pH 4.9 to 5.1; in

* Unpublished data secured by Dr. N. P. Sherwood in this laboratory.

mannite, pH 5.1; in maltose, pH 5.3 to 5.5. This final hydrogen-ion concentration for the various carbohydrates differed to the extent of 0.1 to 0.2 pH in different tests. None of the strains, with the possible exception of No. 5, had a tendency to give a consistently higher or lower hydrogen-ion concentration in any series of determinations. Therefore, there were no differences here which could be correlated with antigenic differences. No. 5, however, consistently produced less acid than the other strains. This strain also differed antigenically. In dextrin the sample of carbohydrate used affected the final hydrogen-ion concentration to a marked degree. An organism giving good acid production in one test might give little acid in a subsequent test. It was impossible to correlate these variations with other characteristics, and it was felt that extraneous factors which could not be controlled were largely responsible for these differences. In the case of xylose fermentation those organisms which attacked xylose readily reached a final hydrogen-ion concentration of pH 5.2 to 5.6 without showing any significant variations between the strains nor any correlation with observed antigenic differences.

One may conclude, then, that the final hydrogen-ion concentration in any one carbohydrate is constant for that carbohydrate, provided that other factors such as the composition of the medium, size of the inoculum, initial acidity and so forth are adequately controlled. This statement applies more particularly to the easily available carbohydrates such as, in the case of *B. typhosus*, dextrose, mannite, maltose, and in the majority of cases xylose, also.

THE AMOUNT OF DEXTROSE NECESSARY TO PRODUCE THE FINAL HYDROGEN-ION CONCENTRATION AND THE INFLUENCE OF BUFFERS AND INITIAL ACIDITY ON THE FINAL HYDROGEN-ION CONCENTRATION.

The amount of sugar necessary to produce the final hydrogen-ion concentration has been investigated by numerous workers. Avery and Cullen (1918), working with human and bovine streptococci, found that the final hydrogen-ion concentration was reached in broth containing 0.5 to 1.5 per cent dextrose. H. Jones (1920) found 0.2 per cent dextrose sufficient to give the characteristic limiting hydrogen-ion concentration for streptococci. Foster (1921*b*) found that 0.3 per cent dextrose was necessary for the production of the final hydrogen-ion concentration in cultures of *Streptococcus hemolyticus*. Browne (1914), using *B. coli*, found that with less than 1.0 per cent dextrose, varying amounts of titratable acid were obtained, but that the amount of acid was quite constant when the

percentage of dextrose was more than 1 per cent. Clark and Lubs (1915) found that *B. coli* required 0.5 per cent dextrose in order to reach its characteristic hydrogen-ion concentration and that if sufficient dextrose was present to give this hydrogen-ion concentration there was no marked alkaline reversion. Fred, Peterson and Davenport (1919) found that *B. typhosus* and other xylose fermenting organisms gave the largest acid yield in 2.0 per cent xylose broth. H. Jones (1920) found that 0.2 per cent dextrose would yield the characteristic final hydrogen-ion concentration with *B. typhosus, B. paratyphosus A*, and *B. vulgaris*. My results do not entirely agree with his. It is possible that his broth required less glucose to reach the final hydrogen-ion concentration because of its lower buffer content. He states that "ordinary broth is used." That this buffer factor is a very important one in bacteriological culture media has long been recognized. Henderson and Webster (1907) suggest the use of phosphates to preserve the neutrality of media during the growth of acid or alkali-forming organisms. Clark (1922*a*) has pointed out the great importance of buffers in culture media. Clark (1915*b*) showed that *B. coli* produces a somewhat higher concentration of hydrogen-ions in more highly buffered media. Kligler (1916), Berman and Rettger (1918), Bronfenbrenner and Schlesinger (1918*b*) have noted that the amount of acid produced depends on the concentration of carbohydrates, buffer and quantity of peptone used. Foster (1921*b*) notes that with dipotassium hydrogen phosphate used as a buffer, *Streptococcus hemolyticus* produced large amounts of acid, but did not reach its final hydrogen-ion concentration with less than 1.0 per cent of dextrose present.

Summarizing my experiment, then, I found that my organisms required from 0.4 to 0.6 per cent dextrose in meat infusion broth to give a characteristic final hydrogen-ion concentration of pH 4.9 to 5.1 in twenty-four hours. This was slightly higher than the figure given by others for various organisms working under different conditions. It is suggested that the natural buffers of meat infusion broth may account for this. In buffered broth from 0.8 to 1.0 per cent dextrose was needed to give the final hydrogen-ion concentration under the conditions of the test. It is impossible to make a close comparison between these results and those obtained with other organisms, as there appears to be little published on this subject.

My results showed that almost twice as much dextrose was needed to give the characteristic hydrogen-ion concentration in buffered as

in unbuffered broth. An adequate explanation of this is that it is due to a suppression of free-hydrogen-ions by the action of the buffer. The organisms used four-tenths to six-tenths per cent dextrose in unbuffered broth before producing the final hydrogen-ion concentration. This is a larger figure than is given by most authors. One explanation of this is that meat infusion broth is a highly buffered medium. Much of the work quoted was done with peptone water. This I have found to be poor in buffers and not a particularly suitable medium for the growth of *B. typhosus*.

H. Jones (1920) notes that the initial acidity has little to do with the final hydrogen-ion concentration, provided that it is in the optimum range for growth. Schoenholz (1921) warns us of the disproportionate changes taking place near the acid or alkaline limits of the hydrogen-ion concentration admitting growth. In my experiments the initial acidities varied from pH 7.0-7.5 but were well within the optimum zone of growth as shown in experiment I.

The initial reaction of the medium had little or no effect on the final hydrogen-ion concentration provided it was within the range of optimum growth.

ACID DEATH POINT.

Work on the acid death point of bacteria has been attacked from two angles—the disinfectant action of acids added to cultures, and the acidity produced by an organism as a result of its own fermentation activities. Winslow and Lockridge (1906) give a comprehensive review of the action of acids and salts as disinfectants. They found *B. typhosus* to be very susceptible to dilute acids and the toxicity of the acid depended on the free H-ions in the case of inorganic acids. Using organic acids, acetic and benzoic, the disinfectant action of the H-ions was complicated by a toxic action due to the anion and undissociated molecules. The presence of peptone decreased the disinfectant action of the acids and the authors suggest it may be due to a loose protein-acid combination. Since this work, a great deal has been done showing that the presence of buffers, such as various proteins and salts, decrease the toxicity of the hydrogen-ions. Bruenn (1915) found that the disinfectant action of lactic and acetic acids for *B. coli* and *B. typhosus* was due to the activity of the hydrogen-ions. Clark (1916) emphasizes the fact that it is the hydrogen-ion concentration and not the total amount of acid that limits growth. Michaelis and Marcora (1912) discuss the limit of acid production by *B. coli* and the maximal acid production which is compatible with life. They also point out that the

injury is due to the hydrogen-ion. They found that acetic acid was toxic in a concentration of from 4.10^{-5} to 8.10^{-5}. McDonald (1917) found that *B. typhosus* could withstand three-tenths per cent of lactic acid. His results are greatly complicated by the addition of glucose to his test medium, and since *B. typhosus* ferments glucose with the production of both lactic and acetic acids, it is hard to evaluate his results. Rambousek (1900) found *B. typhosus* is less resistant to lactic acid than is *B. coli*. Kitasato (1888) recognized that the action of different acids depends on the medium and the age of the culture used for inoculation. Duchacek (1904) found that *B. typhosus* produces about equal quantities of acetic and lactic acids, possibly more lactic acid than acetic. Harden (1901) found that *B. typhosus* produces lactic, acetic and succinic acid, ethyl alcohol and a considerable amount of formic acid. Sera (1910) found that *B. typhosus* produces acetic acid and a trace of alcohol Thus it may be seen that the acids produced by the metabolism of the organisms may have disinfectant action.

My experiments on the viability and the final hydrogen-ion concentration show that where pH 4.9-5.2 is reached the organisms are generally dead in seventy-two hours. This corresponds to the figures given by Ishii (1920) for glucose broth. More of my organisms remained viable after seventy-two hours in mannite than they did in glucose. Sera (1910) has pointed out that *B. typhosus* produces less acetic acid from mannite than from glucose. This may be a factor in the greater viability in mannite.

In maltose, dextrine and xylose the organisms did not produce so much acid and remained viable over greater periods of time. Two factors may be concerned, namely, the greater viability may be due to the smaller concentration of hydrogen-ions or to the production of less toxic by-products. Fred and Peterson (1920) found that *B. typhosus* produced formic, acetic, butyric and succinic acids, ethyl alcohol and a very small amount of CO_2 from xylose. Only twenty-five per cent of the xylose was used. They note that the reason for the use of such a small amount of xylose is not known. Burton and Rettger (1917) suggest that the cessation of growth of organisms is due to the end products or an equilibrium of the enzymes produced. Clark (1915 *b*), Winslow, Kligler and Rothberg (1919), and Browne (1914) point out that some of the limiting factors may be due to toxic products other than acids produced during growth.

My results were examined with a view to ascertaining whether

there were any strains which consistently showed a resistance to the acids produced. With the exception of No. 5, if the strains were able to produce a hydrogen-ion concentration of from pH 4.9 to 5.2 in a fermentable carbohydrate they were not viable after seventy-two to ninety-six hours. No. 5 did not, as a rule, produce as much acid as the other strains, but it was also more acid resistant. This is borne out by the experiment showing the growth range of the different organisms.

It may be seen from the foregoing that in dealing with the limiting hydrogen-ion concentration of any organism, the following factors must be considered: First, the concentration of hydrogen-ion; second, the acids produced by the fermentative activity of the organisms; third, other toxic substances produced during growth.

ALKALINE REVERSION.

A slight but definite change in hydrogen-ion concentration toward the alkaline side was noted in all cultures after seventy-two hours or longer. In those cases where the organisms remained viable, the alkaline reversion was more marked. Kendall (1921) notes this alkaline reversion after death of the organism and states that it is probably due to autolytic changes. It is accompanied by a decrease in protein-nitrogen and an increase in non-protein nitrogen. Hall and Randall (1922) state that a fall in acidity of the medium is not proof that the medium is free of sugar. They suggest that acid may be changed to less dissociable compounds. Fischer (1913) noted this alkaline reversion and suggested that it might be due to binding of the acid or to the production of bases. Ayers and Rupp (1918 *a* and *b*) in their explanation of the methyl-red reaction with *B. coli* and *B. aerogenes*, indicate clearly that a simultaneous decomposition of the acid by products independent of ammonia production may take place with a consequent alkaline reaction.

Levine (1916 *a* and *b*) states that if, after the limiting hydrogen-ion concentration is reached by an organism, it remains viable, it will attack the proteins, forming alkali which neutralizes some of the acid and enables it to use more carbohydrate.

The alkaline reversion considered above should be distinguished from the progressively alkaline reaction produced in a culture medium which contains no available carbohydrate. The tendency to slight alkaline reversion was noted in all the strains and was not correlated with any antigenic differences observed. No. 5, how-

ever, always produced a more marked alkaline reaction than the other organisms.

There was, however, a decided tendency toward a greater alkaline reversion in the order of availability of the carbohydrate used. Dextrose gave the greatest concentration of hydrogen-ions and showed the least reversion, never more than 0.4 pH. Mannite was practically the same as dextrose. Maltose gave a lower concentration of hydrogen-ions and showed a reversion of from 0.4 to 1.0 pH. Dextrin gave the lowest hydrogen-ion concentration values and the reversion varied from 0.6 to 1.9 pH. The rapid xylose-fermenters gave an average pH of 5.2 to 5.6 and an alkaline reversion from 0.3 to 1.0 pH.

No. 5, which produced no acid from xylose, gave a progressively alkaline reaction. The slow xylose-fermenters gave an alkaline reaction, then a slight acid reaction followed by an alkaline reversion. It would seem in this case that the organism was able to use only a very small amount of the xylose and that the alkaline reaction was due to the subsequent utilization of proteins.

SUMMARY.

The findings of the foregoing experiments may be summarized as follows:

1. The range of growth in plain meat infusion broth of the strains of *B. typhosus* used was from pH 5.0 to pH 9.0.

2. There was a slight increase in titratable acidity in broth to which no carbohydrate had been added. The reaction after twenty-four hours became increasingly alkaline.

3. The final hydrogen-ion concentration reached in dextrose, mannite and maltose broth was characteristic for the carbohydrates used and was constant under stated conditions. This was also true for xylose in ninety per cent of the strains. The final hydrogen-ion concentration in dextrin was variable and appeared to depend somewhat upon the solubility of the sample of dextrin used.

4. A definite amount of dextrose was necessary to give the characteristic limiting hydrogen-ion concentration. This amount was larger if a broth rich in buffers was used.

5. The initial hydrogen-ion concentration of the carbohydrate used did not affect the final hydrogen-ion concentration provided the initial hydrogen-ion concentration was in the optimum range of the organisms.

6. In broth containing a fermentable carbohydrate, if pH 4.9-

5.2 was reached, the organisms were rarely viable after seventy-two hours.

7. The kind of carbohydrate fermented determined to a certain extent the viability of the organisms after the final hydrogen-ion concentration was reached.

8. There was a distinct alkaline reversion present in all cultures twenty-four to forty-eight hours after the final hydrogen-ion concentration was reached. This reversion was least apparent in dextrose and increased in the different carbohydrates in the order named —mannite, maltose, xylose and dextrin.

9. No noteworthy differences between the different strains was apparent, nor could any relation be discovered between the reactions studied here and the antigenic differences exhibited by these organisms. No. 5 is an exception to the above statement.

QUANTITATIVE TESTS.—EXPERIMENTS ON THE UTILIZATION OF DEXTROSE AND ITS RELATION TO THE BUFFER CONTENT OF THE MEDIUM; TO THE TITRATABLE ACID; TO THE GROWTH RATE AND THE FINAL HYDROGEN-ION CONCENTRATION.

The importance of the interrelation between the sugar utilized, the buffer effect of the medium and the acids produced has engaged the attention of bacteriologists for many years. Kitasato (1888) recognized that the effect of acids on an organism was greatly influenced by the composition of the medium. Iwanow (1892) using various organisms, studied the formation of acids in milk and peptone-lactose water, finding that there was twice or three times more acid produced in milk than in peptone water.

Recently there has been a great deal of work done on streptococci to determine the final hydrogen-ion concentration. Since this is so intimately bound up with the amount of dextrose used and the buffer effect of the medium these factors are also considered in these articles.

Fuller and Armstrong (1913) found that over 3.5 per cent acid is produced so uniformly by human strains of fecal streptococci that this may serve to distinguish them from fecal streptococci from animal sources.

Jones, F. S. (1918 *a* and *b*), studied the amount of titratable acid in carbohydrate broth and its relation to the source of the streptococci used. The same author (1920) showed that the kind of media used and the buffer content of the media exerted an effect on the limiting hydrogen-ion concentration. He found that peptone greatly

increased the amount of acid produced, probably due to its buffer action.

Ayers and Rupp (1922) found that the hydrogen-ion concentration of the media and the amount of buffer present exerted an effect on the hippurate-splitting enzyme of hemolytic streptococci.

Avery and Cullen (1919 *a* and *b*) showed that 0.5-1.5 per cent dextrose was sufficient to give the final hydrogen-ion concentration of *Streptococcus hemolyticus* in meat infusion broth. No change in reaction occurred after the first twenty-four hours. These same authors (1919) working with pneumococci found that 0.4 per cent dextrose was necessary to give the final hydrogen-ion concentration. The organisms did not reach their final hydrogen-ion concentration in nine hours, but did in twenty-four hours. Lord (1919) showed that there was rapidly increasing acidity in pneumococci cultures accompanied by rapid growth.

Julianelle (1922) found that staphylococci do not use 0.5 per cent dextrose in peptone water containing 0.5 per cent dipotassium hydrogen phosphate. Grace and Highberger (1920) using *Streptococcus viridans* found that the final hydrogen-ion concentration does not vary in a medium containing from 0.2 per cent to 1.0 per cent dextrose.

Ayers, Johnson and Davis (1918) emphasize the fact that titratable acid is influenced so much by the nature and buffer content of the medium that this method should be supplanted by hydrogen-ion determination.

Pere (1892), using *B. typhosus* and *B. coli*, recognized that a lag in acid formation occurred unless the inoculum was young. He found that the amount of acid depended on the amount of sugar present. *B. typhosus* removed ninety per cent of dextrose in a digested egg medium in fourteen days, and *B. coli* did not entirely use 5.0 per cent lactose in eight days.

Tissier (1902) recognized that *B. coli* produced acid from dextrose and that protein was not so readily attacked in dextrose medium. He also states that the alkaline reversion which took place in some cases was due to an insufficient amount of sugar. He estimated that 0.173 per cent normal sulphuric acid was necessary to limit the growth of *B. coli*. He also suggests that certain strains of staphylococci may be able to attack proteins and dextrose simultaneously.

Kendall (1913) points out that the progressive acidity in a carbohydrate-containing medium is due to the utilization of the

carbohydrate for energy by the rapidly growing organisms. If the carbohydrate may be entirely used without producing enough acid to limit the growth of the organism, they then attack the proteins with the production of ammonia and an alkaline reversion of the medium. On the other hand, in a carbohydrate-free medium the only source of energy resides in the protein molecule. The ammonia is split off prior to the utilization of the nitrogen-free remainder of the molecule for energy and the reaction of the medium becomes progressively alkaline. Kendall then points out that fermentation takes precedence over putrefaction.

Observations on the reaction of the medium, therefore, give a certain amount of information concerning the ability of the organisms to utilize carbohydrates. This ability again hinges on the buffer effect of the medium. It has been assumed by some authors that a progressively alkaline reaction presupposed an inability on the part of the organism to utilize the carbohydrate present. That this is not the case is shown by DeBord (1923) in his studies on *B. pyocyaneus*. Quantitative dextrose determination showed a steady diminution in dextrose in spite of the increasing alkalinity of the medium. This alkalinity may be due to the further utilization of the acids produced from the dextrose or to the neutralization of the acids by the basic products of proteolysis. Sherwood (1926), using *B. pyocyaneus*, showed that certain of his strains agreed with the findings of DeBord (1923), but that in a number of strains, glucose utilization was accompanied by increasing acidity. The work of Ayers and Rupp (1918) has also shown that dextrose may continue to be utilized in spite of the production of an alkaline reaction by *B. aerogenes*.

Levine (1916) points out that if the organisms remain viable after the final hydrogen-ion concentration is reached, they attack the protein constituents, forming basic substances which neutralize the acids and allow the formation of more acid from the remaining carbohydrate.

Glenn (1911) found that the amount of acid produced in any medium was the function of the medium rather than the function of the organism.

In the following experiments an attempt was made to correlate the following factors:

1. The rate and total amount of dextrose used by *B. typhosus* in buffered and unbuffered broth.

2. The rate and amount of acid produced.

3. The rate and extent of the change in hydrogen-ion concentration.

4. The rate of growth of *B. typhosus*.

5. Comparison of the metabolism of the strains used to one another and to the serological differences exhibited by these strains as demonstrated in Part I.

METHODS.

In the following experiments determinations of glucose, titratable acidity, number of organisms and hydrogen-ion content were made on a buffered and unbuffered medium at three-hour intervals for twelve hours and thereafter at 24-, 48- and 72-hour periods. Many determinations were done at seven-day and fourteen-day periods, but these are not recorded in the tables, as no significant change occurred after the death of the organisms, which usually took place before the fourth day. If the determinations were continued after the fourth day, it was necessary to weigh the flasks, and after incubation make up the loss due to evaporation by adding distilled water.

MEDIA. The unbuffered medium was meat-infusion broth, to which the dextrose was added in sterile solution.

The buffered medium was meat-infusion broth, to which was added 16 cc. of dihydrogen potassium phosphate and 144 cc. disodium hydrogen phosphate for every 1,500 cc. of medium. The dextrose was added as above.

The broth was adjusted to a slightly alkaline reaction by the colorimetric method. In many cases, the hydrogen-ion concentration was checked by the electrometric method.

The inoculum consisted of 0.2 cc. of a third-generation eighteen-hour broth culture. The number of organisms was determined by plating, in duplicate, 1 cc. of the medium in proper dilutions in plain agar adjusted to pH 7.0-7.5. The plates were incubated for seventy-two hours and the colonies counted. The figures given in each case are the logarithm of the number of organisms found.

The Folin titration method was used at first for the dextrose determinations. This method proved to be troublesome and subject to several sources of error. The broth bumped and spattered badly even when all reasonable precautions were used to prevent this. As the solution can be boiled only six minutes without danger of destroying the sharpness of the end point, it was difficult to do this when the broth spattered so badly. When two or three successive

titrations could be checked within reasonable limits, the results could be accepted, but the technical troubles mentioned made such checks difficult and time consuming. The final method used was the Folin-Wu colorimetric method for blood-sugar determination.

A protein-free filtrate was prepared using the method given for preparation of a filtrate from blood, Cole (1919). Aliquot parts of the clear filtrate were diluted in volumetric flasks, a 1-50 or 1-100 dilution being satisfactory in most cases. Two cubic centimeters of this dilution were placed in Folin tubes with the alkaline-copper solution and treated as for blood filtrates. Two-tenths milligram and four-tenths milligram standard glucose solutions were prepared in the same way. The comparison was made with the most suitable standard in a Dubosque colorimeter. All tests were made in duplicate. A comparison of the titrametric and colorimetric methods showed that the titrametric method was as accurate as the colorimetric method, but in the long run it was more time consuming and because of the difficulties mentioned above it was less satisfactory than the colorimetric method.

The titratable acidity was determined by titration of duplicate or triplicate 5 cc. quantities of the broth diluted with 45 cc. of distilled water. Titration was done with N/20 NaOH, using phenolphthalein as in indicator. The titratable acidity is expressed in number of cubic centimeters of acid per 100 cc. of medium. Berman and Rettger (1918*a*), Jones, F. S. (1920), and others have demonstrated that it is not necessary to subtract the initial titratable acidity from the final titration.

The hydrogen-ion concentration of the broth was determined by the colorimetric method in many cases and in others by the electrometric method. The limits of error between the two methods was never more than plus or minus .1 pH.

RESULTS.

(*a*) *Utilization of Dextrose.*

The following tables give the amounts of dextrose used by those organisms showing the greatest amount of variation. As a rule the differences observed between the different strains were no greater than those observed in different determinations of the same strains.

TABLE XVI.—Utilization of glucose in unbuffered broth.

ORGANISMS.	Original sugar.	0 pH.	3 hours.			6 hours.			9 hours.		
			Sugar.	Sugar used.	pH.	Sugar.	Sugar used.	pH.	Sugar.	Sugar used.	pH.
2	1.219	7.0	1.179	.040	7.0	1.179	.051	6.8	1.004	.219	6.1
5	1.219	7.0	1.136	.083 (6%)	7.1	1.136	.083	6.8	1.037	.183	6.2
1	1.2690	7.0	1.240	.0290	7.0	1.1848	.0842	6.8	1.0822	.1868	6.4
21	1.3513	7.0	1.2690	.0823	7.0	.1904	.1609	6.9	.1106	.2452	6.0
3	1.0460	7.6	1.0282	.0278	7.5	.8417	.2043	5.5	.7418	.3042	5.3
12	1.2690	7.6	1.2315	.0370	7.4	1.1674	.1016	6.8	1.0632	.2058	5.3
13	1.1240	7.8	1.1164 1.1108	(6%) .0076 .0132	7.2	.9192 .9192	(10%) .2208 .2208	6.4	.8954	.2286 (20%)	5.3
4	1.0956	7.6	.9876	.1080 (9%)	7.4	.9756	.120 (10%)	5.6	.8608	.2348 (21%)	5.6
14											

TABLE XVI.—Utilization of glucose in unbuffered broth—*Concluded.*

ORGANISMS.	12 hours.			24 hours.			48 hours.			72 hours.			Growth.
	Sugar.	Sugar used.	pH.	Sugar.	Sugar used	pH.	Sugar.	Sugar used.	pH.	Sugar.	Sugar used.	pH.	
2	.8150	.408	5.7	.6852	.5338	5.1	.6352	.5838	5.1	.6133	.6057	5.3	—
5	.9350	.2840	5.8	.8563	.3627	5.3	.8146	.4044	5.3	.7331	.4859	5.5	+
1	.9804	.2886	5.9	.8143	.4547	5.2	.6495	.6195	5.3	.5896	.6794	5.3	+
21	.8333	.5180	5.6	.7000	.6511	5.1	.6381	.7132	5.1	.5760	.7753	5.3	+
3	.6906	.3554	5.2	.6250	.4210	5.0	.6022	.4438	5.4	—	—	5.4	—
12	.9956	.2734	5.2	.7278	.2884	5.1	.5630	.4532	5.3	.5176	.4986	5.2	—
13	.8950	.2290 (20%)	5.1	.8552	.2680	5.1	.8550	.2682	5.1	.8314	.2918	5.2	—
4	.8080	.2876 (26%)	5.3	.7652	.3304	5.1	.7063	.3893	5.241	.7064	.3892	5.2	—
14				.8230 .8126	.3344	5.0	.7662 .7421	.3999	5.0	.7119 .7168	.4372	5.1	0

TABLE XVII.—Utilization of glucose in buffered broth by three-hour intervals.

ORGANISM.	Original sugar.	0 pH.	3 hours.			6 hours.			9 hours.		
			Sugar.	Sugar used.	pH.	Sugar.	Sugar used.	pH.	Sugar.	Sugar used.	pH.
2	1.1013	7.3	1.0204	.0809	7.3	.6660	.4353	7.2	.5880	.5133	5.6
5	.9048	7.7				.8602	.0446	7.0	.8032	.1016	6.5
1	1.0683	7.5	1.0204	.0434	7.5	.9920	.0718	7.3	.9259	.1379	6.8
21	1.1061	7.3	1.0775	.0286	7.3	.7016	.4045	7.3	.6140	.4921	6.2
3	1.0638	7.5	1.0593	.0045	7.5	.9259	.1379	7.3	.5924	.4714	6.5
12	.8000	7.5	.5680	.2320	7.5	.5400	.2600	7.2	.1106	.6894	6.3
13	.8000	7.5	.5480	.2520	7.5	.4680	.3320	7.1	.0980	.7020	6.2
4	1.080	7.4	1.0524	.0276	7.4	1.000	.0876	7.0	.6152	.4648	5.8

TABLE XVII.—Utilization of glucose in buffered broth by three-hour intervals—*Concluded.*

ORGANISM.	12 hours.			24 hours.			48 hours.			72 hours.			Growth.
	Sugar.	Sugar used.	pH.	Sugar.	Sugar used.	pH.	Sugar.	Sugar used.	pH.	Sugar.	Sugar used.	pH.	
2	.5320	.5693	5.8	.3538	.7475	5.0	.3508	.7505	5.1	.3418	.7595	5.1	0
5	.7016	.2032	6.5	.3498	.5550	5.3	.2596	.6452	5.3	.2450	.6598	5.5	+
1	.5800	.4838	5.7	.3918	.672	5.3	.3538	.710	5.3	.3538	.710	5.3	0
21	.4640	.6421	5.6	.3148	.7913	5.1	.3148	.7913	5.1	.3148	.7913	5.1	0
3			5.3			5.2	.4494	.6144	5.3	.3572	.7066	5.3	0
12	.0586	.7414	5.8	.04410 .0727	.7559 .6077	5.4	.0450 .0454	.7568 .6350	5.6	*	*	5.6	+
13	.0638	.7362	5.8	.0432	.7568 .7252	5.3	.4596	.7252	5.6	.4444	.7404	5.6	+ +
4	.4968	.5832	5.2	.3720	.7080	5.0	.3720	.7079	5.0	.3718	.7082	5.3	0

* Too small to be determined.

Table XVI gives the change in hydrogen-ion concentration and utilization of sugar by three-hour periods for the first twelve hours and thereafter at 24-, 48- and 72-hour periods, using meat-infusion dextrose broth without the addition of phosphates. It will be seen that in most cases the largest amount of sugar was used between the sixth and twelfth hours. Up to the third hour, very little sugar was used; the amount averaged 0.4 gram. From the third to the sixth hour, the consumption increased, giving an average of 0.09 gram used. From the sixth to the ninth hours 0.13 gram was used, and from the ninth to the twelfth 0.1 gram. There was a marked decrease in the consumption of sugar from the twelfth to the twenty-fourth hour, the average amount used for three-hour periods was only 0.01 gram; from the twenty-fourth hour to the seventy-second hour there was usually a small amount utilized, from 0.005 to 0.23 gram, an average of 0.07 gram. Frequently a small amount of sugar was used between the twenty-fourth and forty-eighth hour, an average of 0.05 gram. Very little or none was used between the forty-eighth and seventy-second hour, an average of 0.02 gram. Strain No. 5 used from 0.04 to 0.05 gram between the twenty-fourth and forty-eighth hour, and from 0.08 to 0.12 gram between the forty-eighth and seventy-second hour as against 0.07 gram for the other organisms.

As long as the organisms remain viable there was a small amount of sugar used. Presumably this sugar served as a source of energy. The organisms after twenty-four hours were decreasing in numbers and only the minimum amount of carbohydrate was required. Except for strain No. 5 there were no constant differences observed between the organisms which could be correlated with their antigenic differences.

Table XVII shows the utilization of dextrose in a heavily buffered meat-infusion broth. Here there was greater irregularity in the rate and amount of sugar utilization. The amount of sugar used in the first three hours was greater than in the unbuffered broth. The amount used between the third and sixth hours was also greater, average 0.13 gram. Maximum utilization usually took place between the sixth and ninth hours in the buffered as well as in the unbuffered broth, an average of 0.23 gram being used: that is, almost twice that used in unbuffered broth. From the ninth to twelfth hours the amount of dextrose used decreased to 0.11 gram. The decrease in sugar utilization was therefore greater in the buffered than in the unbuffered medium. From the twelfth to the twenty-

fourth hour, the average consumption of sugar for three-hour periods was 0.04 gram. This was four times the amount per hour used by the organisms in unbuffered broth. From the twenty-fourth to the forty-eighth hour, very little dextrose was used, average 0.02 gram; that is, about one-half that used in unbuffered broth. There was practically no dextrose used between the forty-eighth hour and the seventy-second hour. A comparison between the average amount of dextrose used in the buffered and unbuffered broth by three-hour periods showed that the total quantity used in buffered broth was considerably greater than in unbuffered broth. The amount used in the first three hours was much greater in the buffered broth, while during the next three hours the amount used in buffered broth was only slightly greater than in unbuffered broth. The maximum utilization of dextrose in both buffered and unbuffered broth occurred between the sixth and ninth hours but the total utilization in buffered broth was greater during this period, being two and a half times that in unbuffered broth. The inhibition in dextrose utilization from the ninth to twelfth hour was more marked in buffered than in unbuffered broth. There was less utilization of dextrose after the twenty-fourth hour in buffered broth. This was probably a continuance of the inhibition noted after the ninth hour.

The average for these results may be seen in Table XVIII. The total amount of dextrose used in buffered and unbuffered broth is an excellent example of the effect of buffers on the utilization of dextrose. The organisms used from 75 to 100 per cent of the dextrose present in buffered broth, but only from 25 to 50 per cent of the dextrose in unbuffered broth.

As in unbuffered broth, no constant differences were observed in the dextrose utilization by the different strains, nor was there any correlation between the utilization of dextrose in buffered broth and antigenic differences with the exception of strain No. 5.

(b) Change in Titratable Acidity and Hydrogen-ion Concentration in Buffered and Unbuffered Broth.

Tables XIX and XX give the change in titratable acidity and hydrogen-ion concentrations of the organisms at three-hour intervals and thereafter at 24,- 48- and 72-hour periods in buffered and unbuffered broth.

The greatest increase in titratable acidity took place during the period when the dextrose was being most rapidly used. There was a slight increase in acidity up to the third hour. From the third to

the sixth hour the average increase was 1.62 per cent; from the sixth to the ninth hour 2.35 per cent, and from the ninth to the twelfth hour 0.91 per cent. From the twelfth to the twenty-fourth hour there was an increase in acidity averaging 0.19 per cent for each three-hour period. The increase in titratable acidity in the unbuffered broth up to the twenty-fourth hour was parallel to the utilization of dextrose. The changes in titratable acidity from the twenty-fourth to the seventy-second hour were somewhat irregular. In about fifty per cent of the determinations a slight decrease in titratable acidity took place between the twenty-fourth and the seventy-second hour. This decrease was more marked when the observations extended over a week. It was probably due to autolytic changes, since with the exception of No. 5 all of the organisms in unbuffered broth were dead by the seventy-second hour. The final reaction of the medium was from 6 to 8 per cent acid to phenolphthalein after twenty-four hours.

The change in hydrogen-ion concentration in unbuffered broth followed the change in titratable acidity and sugar utilization closely from the third to the twelfth hour, when the final hydrogen-ion concentration was usually reached. There was little or no change in hydrogen-ion concentration up to the third hour although dextrose was being used during this period. After the twelfth hour the hydrogen-ion concentration remained constant, although dextrose was being used and titratable acid was increasing. After twenty-four hours a slight alkaline reversion took place and was usually accompanied by a decrease in titratable acidity. Using strain No. 5, the decrease in titratable acid and the decrease in hydrogen-ion concentration was more apparent than with the other organisms. This organism was usually viable after seventy-two hours and continued to use dextrose in spite of the alkaline reversion. Aside from this there were no peculiarities noted for any of the individual strains used.

TABLE XVIII.—Analysis of percentage of sugar used in buffered and unbuffered broth by hours.

ORGANISM.	3 hours.		6 hours.		9 hours.		12 hours.		24 hours.		48 hours.		72 hours.	
	B	P	B	P	B	P	B	P	B	P	B	P	B	P
2	7%	3	30	4	46	17	51	33	67	44	68	49	68+	49+
5	—	6	4.9	7	11	14	22	23	61	30	71	—	72	39
1	4	2	6	6	13	14	45	22	63	35	66	—	66+	53
21	2 7	6	36 10	11	44 32	18	58 71	38	71 81	49	— 83	—	— 84	58
3	4	2	13	19	44	29	—	33	—	40	57	42	66	54
12	29	2	32	15	86	31	92	36	94 89	36	94 93	36	— 93	37
13	31	0.6	41	10	87	20	92	20	61	23	61+	23	62	25
4	2	9	8	10	43	21	54	26	65	31	65	31	65	35
Average	10	3.8	20	9.6	45%	20	60%	28	72	36	73	36	75	42

B.—Buffered broth.
P.—Unbuffered broth.

TABLE XIX.—Rate of acidity in glucose unbuffered broth.

ORGANISM.	Original number of organism.	pH.	3 hours.			6 hours.			9 hours.		
			Acid.	pH.	Organism log.	Acid.	pH.	Log.	Acid.	pH.	Log.
2	3.9031	7.0	2.2	7.0	4.0000	2.3	6.8	9.3010	5.22	6.1	9.6385
5	4.3979	7.4	2.02	7.3	6.2900	4.1+	6.8	8.2553	5.80+	5.7	9.0000
1	4.8451	7.4	2.80	7.4	5.3222	3.15+	6.7	8.3010	5.45	5.3	8.4472
21	6.4014	7.5	2.10	7.5	6.6021	2.37	7.1	7.3002	4.72	.....	8.8415
3	6.4281	7.5	2.00	7.5	6.6021	4.62+	6.5	7.4771	6.82	5.8	9.5682
12	4.7709	7.6	.7+	7.4	4.771	4.5+	6.8	6.6532	5.7	5.3	6.9542
13	5.6990	7.8	2.00	7.2	6.8808	4.0+	6.4	7.3010	6.7	5.3	8.4771
4	.21 3.9031	7.4	2.20	7.3	4.0000	2.6	6.7	9.3010	5.22	5.6	9.6385

TABLE XIX—Rate of acidity in glucose unbuffered broth—*Concluded.*

ORGANISM.	12 hours.			24 hours.		48 hours.		72 hours.		Growth.
	Acid.	pH.	Log.	Acid.	pH.	Acid.	pH.	Acid.	pH.	
2	6.67	5.7	10.7924	7.37	5.1	6.25	5.4	6.15	5.4	0
5	5.95	5.3	10.6532	6.50	5.3	7.14	5.3	7.00	5.3	0
1	6.65	5.2	9.0212	7.05	5.2	6.25	5.2	6.82	5.3	0
21	7.2+	5.3	11.6990	8.07	5.1	6.52	5.3	7.45	5.5	+
3	6.87	5.2	12.5416	7.37	5.0	7.20	5.2	6.5	5.2	0
12	7.4	5.2	8.8451	8.50	5.0	—	—	8.0	5.0	0
13	7.5	5.1	10.3010	7.50	5.1	—	—	7.62	5.2	0
4	6.67	5.3	10.7924	7.27	5.1	6.25	5.1	6.15	5.3	0

TABLE XX.—Rate of acidity in glucose buffered broth.

ORGANISM.	Original number of organism.	Original pH.	3 hours.			6 hours.			9 hours.		
			*Acid.	pH.	Organism log.	Acid.	pH.	Log.	Acid.	pH.	Log.
2	5.4624	7.3	2.5	7.3	5.7782	4.35	7.2	6.3032	12.10	6.6	9.1206
5	4.000	7.9	3.05		5.4800	3.25 3.05	7.0	7.4472	7.90	6.5	8.7543
21	5.8096	7.5	.212	7.2	6.0969	4.12	7.2	6.3892	4.87	6.2	9.7493
1	4.0170	7.8	1.5	7.3	5.4800	3.85	6.8	6.3892	9.2	6.7	9.5465
3	5.4624	7.5	2.50	7.5	5.7782	4.35	7.3	6.3032	12.10	5.5	9.4206
12	5.6232	7.8	2.20	7.8	5.3424	2.42	7.5	6.3010	10+	6.3	9.3010
13	5.4624	7.8	2.50	7.8	5.7709	4.75	7.1	6.2553	14.10	6.2	9.0934
4	6.0000	7.4	2.8	7.4	6.3979	4.95	7.0	7.4065	17+	5.8	7.415

* Acidity in terms of 2 per cent normal broth.

TABLE XX.—Rate of acidity in glucose buffered broth—*Concluded.*

ORGANISM.	12 hours.			24 hours.		48 hours.		72 hours.		Growth.
	Acid.	pH.	Log.	Acid.	pH.	Acid.	pH.	Acid.	pH.	
2	18.20	5.8	9.9961	21.2	5.0	21.0	5.1	20.5	5.1	0
5	13.17	6.5	9.5079	20.95	5.3	21.12	5.3	23.60	5.5	+
21	23.05	5.2	10.6990	25.00	5.0	24.12	5.0	20.82	5.1	0
1	14.9	5.3	9.9547	19.50	5.3	20.3	5.3	21.45	5.3	0
3	18.20	5.3	9.9961	21.2	5.2	21.0	5.2	20.5	5.3	0
12	12.0	5.8	10.6990	16.28	5.4	20.55	5.6	20.60	5.6	+
13	16.2	5.8	10.6990	23.75	5.3	20.00	5.6	20.05	5.6	+
4	18.0	5.2	8.959	21.0+	5.0	19.3	5.1	19.80	5.5	0

The titratable acidity in buffered broth also followed the utilization of dextrose very closely. Up to the third hour there was slight increase in titratable acidity; from the third to the sixth hour there was an average increase of 1.5 per cent; from the sixth to the ninth hour, that is, during the period of maximum sugar utilization, there was an average increase of 6.7 per cent; from the ninth to the twelfth hour, an increase of 5.7 per cent, and from the twelfth to the twenty-fourth hour an increase of 4.5 per cent. From the twelfth to the twenty-fourth hour, the average increase for each three-hour period was 1.1 per cent. During this period, then, there was apparently a disproportionate amount of acid present. This is also noted by Jones, F. S. (1920). The dextrose is being used in diminishing amounts, but the acids continue to accumulate in the medium. From the twenty-fourth to the seventy-second hour there was little or no increase in titratable acidity. This seems to be due to the fact that very little dextrose was being used during this period.

Strain No. 5 continued to show an increase in titratable acidity up to the seventy-second hour in buffered broth with a gradual decrease from this time on. The utilization of dextrose by No. 5, with an increase in titratable acidity, continued for a longer period of time in buffered than in unbuffered broth.

With all strains the change in hydrogen-ion concentration was much slower in buffered than in unbuffered broth. The change in hydrogen-ion concentration up to the third hour was not appreciable, while the change from the third to the ninth hour was less than that which occurred in unbuffered broth. The greatest change occurred during the period of maximum utilization of dextrose and acid production, that is, from the sixth to the ninth hours.

The final hydrogen-ion concentration was not reached in twelve hours as a rule, a drop from 0.1 to 1.2 pH being noted between the twelfth and twenty-fourth hours. The final hydrogen-ion concentration reached was lower than in unbuffered broth. A larger number of organisms were viable in seventy-two hours, and there was a greater degree of alkaline reversion in buffered broth. In many cases this alkaline reversion was accompanied by an increase in titratable acidity. This might be explained by the union of basic substances with the acids to form an alkaline-reacting compound. This would be accompanied by a decrease in free hydrogen-ions, but an increase in titratable acidity. This alkaline reversion with an increase in titratable acidity was especially marked in the case

of strain No. 5. This organism also remained viable over a longer period of time than the other organisms. No significant individual differences were noted, and there was no parallelism between the metabolic changes and antigenic differences.

(c) *Growth Rates.*

Tables XIX and XX show the growth rates of the organisms under the conditions given above. Buchanan (1918) divided the growth period of an organism into seven phases, namely: First, initial stationary period; second, lag phase; third, maximum or logarithmic increase; fourth, decreasing rate of multiplication; fifth, maximum stationary period; sixth, accelerated death; seventh, death at a constant rate. Chesney (1916) divided the growth period into four phases, namely: First, initial stationary period; second, lag, or growth at slowly increasing rate; third, maximum period; fourth, decreasing rate.

The lag phase has occupied the attention of several investigators. Rahn (1906) noted that the greater the inoculum the shorter the lag. He was not able to get rid of the lag phase entirely, however. Müller (1896) noted that with *B. typhosus* the lag varied with the age of the inoculum and considered that it was due to the injury suffered by the bacteria in the previous medium. Barber (1908) was able to eliminate the lag phase entirely by transferring organisms during the period of logarithmic increase.

If one follows Chesney (1916), an examination of my tables shows that when the first determination was made three hours after inoculation, the stationary period did not appear. The lag phase occurred in the unbuffered broth during the first six hours as a rule, though growth at an increasing rate usually occurred from the third to the sixth hours. The period of maximum growth occurred from the sixth to the ninth hour, while from the ninth to the twelfth hour the organisms were multiplying at a decreasing rate. As no consistently satisfactory counts were obtained on 24-hour samples, the last phases do not appear in the tables. It may be seen, then, that the growth rate calculated in logarithms paralleled dextrose utilization and acid production.

In buffered broth the three-hour periods used were usually too few to distinguish sharply between the lag phase and growth at increasing rate. It is evident, however, that the period of maximum growth occurred between the sixth and ninth hours. The amount of growth taking place between the sixth and ninth hours was

greater in buffered than in unbuffered broth. From the ninth hour, the same increase at diminishing rate was observed as in the unbuffered broth.

The increase in the number of organisms was greater during the period of logarithmic increase in the buffered broth than in the unbuffered, but the levels reached were seldom higher in the buffered broth. There was a tendency for the depression in growth rate to occur earlier in the unbuffered than in the buffered broth.

During the period of maximum growth, from the sixth to the ninth hour, the hydrogen-ion concentration average was from pH 6.7 to pH 5.5 in unbuffered broth and from pH 7.1 to pH 6.3 in buffered broth. One might expect, therefore, to find inhibition beginning before the ninth hour in unbuffered broth. Inhibition was noted in both buffered and unbuffered broth at the ninth hour, being somewhat more marked in the former. This would indicate that factors other than the hydrogen-ion concentration were concerned.

(d) Rate and Relation of the Utilization of Dextrose to the Final Hydrogen-ion Concentration and Titratable Acidity, Inhibition and Death.

A comparison of the utilization of dextrose, titratable acidity, change in hydrogen-ion concentration and growth rates calculated as logarithms of the actual number of organisms in buffered and unbuffered broth shows that in general, they tend to parallel each other. There are certain differences, however, that may be noted. The sugar utilization, titratable acidity and growth rates were much more nearly parallel than were these changes with the change in hydrogen-ion concentration. The buffer effect of the medium seemed to suppress the change in hydrogen-ion concentration, so that in spite of the utilization of dextrose and the accumulation of acids there was very slight change in the hydrogen-ion concentration. The inhibition beginning at the ninth hour also would not be apparent if hydrogen-ion concentration determinations only were done. In unbuffered broth the final hydrogen-ion concentration was reached by the twelfth hour, but there was considerable utilization of dextrose and accumulation of acids as long as the organisms were viable. In buffered broth there was an appreciable change in hydrogen-ion concentration from the twelfth to the twenty-fourth hour, but this change did not give one an idea of the amount of dextrose used.

It was also noted that although the amount of dextrose used and

the growth rate in logarithms were roughly parallel in both buffered and unbuffered broth, the amount of dextrose used per organism was much greater during the first three hours than at any subsequent time. After the third hour the amount used per organism decreased steadily until between the ninth and twelfth hours the amount of dextrose used per organism was inappreciable. Table XXI brings out these facts clearly. It is realized that these figures are approximate rather than exact. Determinations at shorter intervals of time would be necessary to determine exactly the amount used per organism. However, we feel that these figures are as exact as the common use of logarithms to indicate the actual number of organisms present. It seems to be sufficiently exact to indicate the striking difference between the amounts of sugar used per organism at different times during the twelve-hour period.

TABLE XXI.—Utilization of dextrose per organism.

BROTH.	HOURS.	Dextrose used in grams.	Dextrose used per organism.
Unbuffered	3	.01	.000,000,08
	6	.10	.000,000,000,5
	9	.18	.000,000,000,6
	12	.27	.000,000,000,2
Buffered	3	.07	.000,000,056
	6	.10	.000,000,032
	9	.32	.000,000,000,050
	12	.68	.000,000,000,013

The organisms in unbuffered broth used the largest amount of dextrose per organism during the first three hours. The amount used during the next three hours was one one-thousandth of that used during the first three. From that time on, there was very little dextrose used per organism although the maximum rate of reproduction usually started about the sixth hour.

In buffered broth during the first three hours there was a maximum utilization of dextrose per organism. During the next three hours there was only a slight decrease in utilization per organism. Between the sixth and ninth hour there was noted the same enormous drop in the amount of dextrose used per organism despite the fact that here, too, maximum reproduction of the organisms was initiated at the sixth hour. The utilization of dextrose per or-

ganism in buffered broth was much less than in unbuffered broth from the sixth to the twelfth hour. It will be noted also that although the total amount of dextrose used in buffered broth was much greater than in unbuffered, the actual increase in numbers of organisms was about the same. This evidently indicates that in buffered broth each organism was capable of metabolising more sugar than in unbuffered broth.

DISCUSSION.

A discussion of the foregoing results brings up the following questions:

First, the amount of dextrose which may be used by an organism and the influence of the medium upon this utilization.

Second, a comparison of the utilization of dextrose, change in titratable acid, hydrogen-ion concentration and growth rate.

Third, the influence of the products of dextrose metabolism on the activity of the organisms.

Fourth, constancy of the changes exhibited by *B. typhosus* and a comparison with other organisms.

Comparatively few authors have followed the quantitative utilization of dextrose hour by hour, though many have determined the minimum amount of dextrose necessary to give the final hydrogen-ion concentration.

Duchacek (1904 *a* and *b*), using peptone water plus 1 per cent dextrose, found that *B. typhosus* used 18 per cent of the dextrose in eleven days and 41 per cent in thirty days. The total acidity was calculated by adding together the different fractions of the acids determined by chemical analysis. He noted that the total amount of acid as determined by titration was more than can be accounted for by the acids found. It is difficult to see why there should be so much sugar used between the eleventh and thirtieth days since in my experiments and those of other observers on the colon-typhoid group the organisms were seldom viable after ninety-six hours in dextrose broth.

Cole and Onslow (1916) state that *B. typhosus* gave an alkaline reversion with 0.15 per cent dextrose but remained permanently acid in 0.2 per cent dextrose. That this would be true only in a medium low in buffer content may be seen from my tables. Sears (1917) found that *B. typhosus* used 0.138 to 0.270 gram of dextrose on the first day in broth and 0.399 to 0.487 gram on the eighth day. Here again, the effect of an abundant buffer may be noted. This result

agrees with mine except that usually more sugar was used during the first twenty-four hours.

Kligler (1916) using ammonia production as an index of protein metabolism, noted that no ammonia was produced by *B. typhosus* in five to seven days where 0.5 per cent of dextrose was present. The effect of buffers was noted, and he found that where a buffer was present the final concentration of hydrogen-ions in the medium was lower. *B. typhosus* reached pH 4.6 to 4.8 in unbuffered peptone water and pH 4.8 to 5.1 in peptone water buffered with phosphates. This is in accord with my results. My results in buffered and unbuffered broth are in entire agreement with Clark (1915*a*), Bunker (1919), Burton and Rettger (1917), Schoenholz and Meyer (1919), Bronfenbrenner (1918*b*) and Salter (1919), who found in general that the presence of buffers stimulates growth, prolongs the life of the organisms and greatly increases the amount of dextrose used. The results obtained in my experiments indicate that the amount of dextrose used by *B. typhosus* was so dependent upon the buffer content of the medium that it would always be necessary to know the kind of medium used and its buffer content before any comparison could be made with the work of other authors on *B. typhosus* or other organisms.

A number of authors have assumed that the dextrose utilization, change in titratable acidity, growth rates, and change in hydrogen-ion concentration, parallel each other so closely that by following the change in hydrogen-ion concentration, the other changes mentioned will be sufficiently revealed.

Kligler (1916) states that the hydrogen-ion concentration serves to show the amount of sugar which an organism can use without sufficient acid to limit growth and also the rate at which the sugar is utilized. Foster (1921*b*), Anderson (1920), Cullen and Chesney (1918) and Lord and Nye (1919) assume that the above factors are parallel.

In my results the utilization of dextrose and increase in titratable acidity paralleled each other very closely. The lag period, period of maximum increase in number of organisms and period of decrease coincided almost exactly with the utilization of dextrose and increase in titratable acidity when the growth rates were followed by means of the logarithms of the actual number of organisms. However, if the dextrose used per organism was calculated it will be seen that the amount used was very much greater during the lag period than

during the period of maximum reproduction. It might be suggested that this could be due to a storing up of the dextrose in some form in the bodies of the bacteria for the purpose of release during the time of active reproduction. This might be effected by an enzyme. This suggestion is borne out by the smaller accumulation of acid during this period than during the period of maximum growth. In comparing the amount of dextrose used per organism in buffered and unbuffered broth, it will be seen that much more dextrose per organism was used during the lag period in buffered broth. This might be due to the stimulating effect of the buffer on the storage of the carbohydrate in each organism or the lack of buffer might decrease the enzyme causing the storage.

The changes in hydrogen-ion concentration do not follow the dextrose utilization as closely as does the change in titratable acidity, since during the first three hours there was no apparent change in hydrogen-ion concentration. The titration of acids, or the quantitative determination of sugar shows that an appreciable change has taken place. Since little or no change takes place in hydrogen-ion concentration after the final concentration is reached, it might be assumed that no changes occurred. That this is not the case is shown by the above determinations. It is not safe, therefore, to assume, as Chesney (1916) does that because there is no change in hydrogen-ion concentration there is also no metabolic activity going on.

DeBord (1923) using *B. subtilis, B. pyocyaneus, B. coli Cl. botulinum* and *Cl. sporogens,* found that sugar utilization and change in hydrogen-ion concentration paralleled each other closely in those organisms which fermented dextrose with acid production. *B. subtilis* and *B. pyocyaneus,* however, showed increasing alkalinity in spite of utilization of the sugar.

Foster (1921*b*) has divided the change in hydrogen-ion concentration produced by streptococci into five periods, namely: First, stationary period; second, lag or acid at increasing rate; third, acid at maximum or logarithmic rate; fourth, acid at decreasing rate; fifth, stationary period. The changes in hydrogen-ion concentration observed by me would also fall into these five periods, but in dextrose and titratable acid determinations the first period was not noted. The fourth period, acid at decreasing rate, continued from the ninth to the twenty-fourth or forty-eighth hour. The fifth period did not appear as long as any organisms were viable.

I have found, in agreement with most observers, that the period of

logarithmic increase and the maximum utilization of dextrose are parallel. Jones (1920), using pneumococci, showed that the maximum period of growth occurred near neutrality, but that sugar utilization was greatest (as indicated by the change in hydrogen-ion concentration) after the maximum period was ended, that is, during the phase of negative acceleration.

Cohen and Clark (1919) noted the same phenomenon with *B. coli* and suggested that the effect of acidity on fermentation and on growth are independent. My results indicated the reverse of this, since the maximum amount of dextrose used per organism was greatest before the initiation of the period of maximum increase.

It is generally assumed that the maximum utilization of dextrose takes place during the period of logarithmic increase, and this appears to be the logical assumption, since organisms are generally believed to use dextrose for the production of energy. It would appear from my findings that the organisms stored up energy by the utilization of dextrose and then used this stored energy during the period of most rapid multiplication, instead of breaking down the dextrose available in the medium. The apparent parallelism between the period of maximum utilization of dextrose as a whole and the period of logarithmic increase is due to the very large number of organisms present during this period, each one of which is using a very slight amount of dextrose.

Recently it has been assumed that the inhibition and death of organisms in a medium containing a utilizable carbohydrate was largely due to the effect of the hydrogen-ions. There can be no doubt that they play a prominent role. Cohen and Clark (1919), however, noting the early inhibition of the growth of *B. coli* in plain broth where there was no appreciable change in hydrogen-ion concentration, warn against laying too much stress on the toxic effects of the hydrogen-ions. My results show that in unbuffered broth inhibition took place at the ninth hour with an average pH of 5.5. In buffered broth the same or sometimes even greater degree of inhibition took place with a pH of 6.3. It would seem from these results that the great accumulation of acids and other products of metabolism, as well as the hydrogen-ion concentration, exerted an effect on the growth of the organisms.

The constancy of the utilization of dextrose and the other factors here discussed for the strains of *B. typhosus* used has been remarkable. No constant differences between the typical strains of *B. typhosus* were noted with the exception of No. 5. There was no cor-

relation between the serological differences and dextrose metabolism. It is practically impossible to compare the results given here with those of other authors because of the variety of the media used. It would seem, however, that streptococci use less sugar than *B. typhosus*, *B. dysenteriæ* about the same, the paratyphoids and *B. coli* slightly more and *B. aerogenes* and *B. cloacæ* decidedly more than *B. typhosus*.

SUMMARY.

The following gives a summary of the results obtained:

1. The utilization of dextrose was largely dependent on the buffer content of the medium. Buffers stimulated the utilization of dextrose per organism to a marked degree, but the total number of organisms was little if any greater in buffered than in unbuffered broth.

2. The utilization of dextrose appeared to be a function of living cells. The maximum dextrose utilization per organism took place during the lag period. This was followed by the period of maximum growth, during which little dextrose per organism was used. The increase in titratable acid coincided in quantity and rate with the utilization of dextrose.

3. The change in hydrogen-ion concentration did not serve as an accurate indicator for the amount of dextrose used, since there was little or no change during the first six hours of growth, although an appreciable amount of dextrose had been used with a corresponding accumulation of acids. The hydrogen-ion concentration remained stationary after the final concentration was reached while dextrose utilization continued as long as the organisms were viable.

4. The titratable acidity gave a more accurate indication of the amount of dextrose used than did the change in hydrogen-ion concentration.

5. Alkaline reversion to a slight degree after the final hydrogen-ion concentration was reached did not signify complete utilization of the dextrose.

6. Death occurred partly as a result of the toxic effects of the free hydrogen-ions and partly from the accumulation of other toxic growth products.

7. The variations between the strains used were not of any significance except in the case of No. 5, and could not be correlated with antigenic differences.

DISCUSSION OF IRREGULAR STRAINS.

Before proceeding to a discussion of the results here presented, it might be of interest to note the irregularities observed in certain strains of the organisms used. Strains Nos. 21, 40, and 12 (Rawlings) were apparently typical strains of *B. typhosus*, serologically, reacting in high titre with the various sera used, and all belonging to one group as shown in Part I. These strains fermented xylose slowly. Nos. 21 and 12 gave acid after fourteen to twenty-one days. No. 40 gave a very slight amount of acid, pH 5.8 after three weeks. All of these organisms gave daughter colonies on xylose eosin-methylene-blue plates but it was not possible to select rapid fermenters from these colonies. These organisms have been grown in the presence of xylose on plates and in xylose broth for six weeks without acquiring the ability to ferment xylose more rapidly. No. 12 produced a small amount of acid in one hundred and twenty hours, but on transferring to fresh broth it produced no acid until the seventh day.

In arabinose, raffinose, dulcite and rhamnose these organisms never produced acid. Daughter colonies were observed in the case of No. 40 in arabinose, none were formed in dulcite and all formed daughter colonies in rhamnose. No fermenting strains could be picked from these colonies.

No. 5 was an organism which exhibited some of the cultural irregularities noted for the typical strains of *B. typhosus* considered above, and in addition it appeared to be entirely inagglutinable. In an effort to identify it, many different antityphoid sera have been used with entirely negative results. *B. paratyphosus B*, *B. paratyphosus A*, *B. pullorum* and *B. sanguinarium* antisera have been used with equally negative results. No drop in titre for the homologous organisms resulted when the above antisera were absorbed by No. 5. Antiserum prepared by the injection of No. 5 into rabbits agglutinated No. 5, but did not agglutinate any of the typical strains of *B. typhosus* nor *B. paratyphosus A* or *B*, *B. Flexner* or *Shiga dysenteria*. Cultures picked from separate colonies at different times have never yielded agglutinable strains. The serum of the patient from whom this organism was isolated did not give a positive Widal. The cultural reactions of No. 5 were also somewhat irregular in the following respects: there was slow but definite blackening of lead-acetate agar; a rapid return to alkaline in litmus milk; fermentation in rhamnose and no fermentation in xylose. In twenty-four hours the rhamnose eosin-methylene-blue plates were negative, in forty-

eight hours the primary colonies showed small but dark daughter colonies, and in seventy-two hours all the colonies were quite dark with a greenish sheen. It was not possible to select daughter colonies producing typical acid colonies in twenty-four hours. In xylose eosin-methylene-blue plates in seventy-two hours, faintly dark daughter colonies were visible. Organisms picked from these daughter colonies did not ferment xylose. Growth for six weeks in xylose broth did not enchance the power of this organism to ferment xylose. In all cases, the reaction was alkaline in two per cent xylose broth during a period of twenty-five days.

This organism also differed in the rate and quantity of dextrose used. Repeated tests showed that it reached pH 5.0 to 5.3 in twenty-four hours, at the end of a week it was viable and still using sugar, but an alkaline reversion had taken place which reached pH 5.5. During the second week, the hydrogen-ion concentration remained practically stationary and very minute amounts of sugar were used. The organisms were frequently dead at the end of the second week. This organism, then, showed a type of sugar metabolism which resembled *B. ærogenes* or *Morgan's bacillus*, since it continued to use dextrose in spite of an alkaline reversion.

No. 5 also had a wider range of growth than the other organisms studied, being viable at pH 9.6. In plain broth adjusted at pH 5.0 the broth was sterile in twenty-four hours, in one per cent dextrose broth, however, although a reaction of pH 5.0 was often reached in twenty-four hours, the organism continued to live and within forty-eight hours was beginning to give an alkaline reaction in the culture medium.

The identification of this organism involved many considerations. The ability of an organism to gain or lose fermentative powers or antigenic properties by natural or artificial means involved the question of the reliability of fermentation and antigenic reactions and also the question of mutation in bacteria. A survey of the literature bearing on these questions revealed many conflicting opinions. Winslow *et al.* (1919), after a comprehensive review of the variations observed in fermentation reactions, reach the conclusion that in the great mass of colon-typhoid strains cultivated under standard conditions the fermentative characters exhibit a high degree of correlation with other biochemical, serological and pathogenic properties. The fermentation or nonfermentation of lactose, sucrose and xylose seem to be especially important and stable characters. Krumwiede (1918), on the other hand, states that variations in physiological

function of bacteria occur with frequency, while general experience indicates a high degree of stability as regards the antigenic properties.

Many variations both spontaneous and induced are recorded in the literature. Thro (1914) concluded that carbohydrate fermentation could not be used to separate strains of streptococci since different colonies picked from a plate streaked from a single colony gave great variations in acid production. Broadhurst (1915) observed spontaneous variations in the direction of the fermentation of carbohydrates not previously fermented. The carbohydrates which showed the greatest variation were those less frequently fermented by streptococci, namely mannite and inulin. Glenn (1911) by plating was unable to select colonies which gave large and small amounts of acid uniformly. Raubitschek (1913) observed variation in acid production, using strains isolated from the different organs of a typhoid cadaver, basing his conclusions on "strong red" and "weak red" reactions in litmus broth medium.

Many authors have reported on the variations produced by artificial means. The best example of this is probably the work of Twort (1907) who induced lactose fermentation in a strain of *B. typhosus* by long cultivation in the presence of lactose. Penfold (1911, 1912) confirmed and extended Twort's work. He reported that lactose-fermenting strains threw off nonfermenting mutants. Many of the fermenting and nonfermenting mutants were picked from daughter colonies. Muller (1911) had observed daughter colonies on rhamnose, but he apparently did not observe acid formation in these colonies, as he suggested that they might be organisms which were capable of using this pentose without acid formation. Teague (1920), Teague and Morishima (1921) and Morishima (1921), reported acid formation in daughter colonies in xylose with strains of *B. typhosus* which fermented this pentose slowly, and also in rhamnose, raffinose and arabinose. In the last-mentioned carbohydrates there was no acquisition of new fermentative power on transfer into broth. The slow xylose-fermenters could be changed into rapid fermenters by this means, however. There was no correlation between xylose fermentation and serological reactions. Teague (1920) concluded that these irregular fermentations were not evidence against the homogeneity of *B. typhosus*.

The occurrence of spontaneous variation in agglutinability in *B. typhosus* has been reported by Weiss (1917), Mock (1919), Durham (1901), Burri and Dugeli (1919), Raubitschek (1913), Toyoda

(1922), and others. Recently several authors have tried to induce antigenic variations by various means. Gardner and Walker (1921) by plating were able to select colonies which varied in antigenic properties. These variations were not stable, however. Arkwright (1921) considered that any culture may contain strains varying in their agglutinogenic powers. The temporary preponderance of one or the other agglutinogenic property in the mass of the population of a particular culture determines the serological character of that culture. Jordan (1926) has reported the appearance of two antigenically dissimilar strains from a single cell line. The antigenic peculiarities were associated with the formation of rough and smooth colonies on agar plates. Blankenhorn *et al.* (1923) reported the isolation of an atypical *B. typhosus* from a periosteal abscess. The patient's serum agglutinated the isolated organism in dilutions of one to eight but did not agglutinate stock cultures of *B. paratyphosus A*, B or *B. typhosus*. Serum prepared from this organism agglutinated the Rawlings strain of *B. typhosus* in low dilutions and slowly. It was also irregular in its cultural reactions in that it did not ferment mannite nor dextrin. It produced slight acid in xylose. Lead acetate was not mentioned. They considered that this organism was probably influenced by its long existence in the body of the patient.

Benians (1920) produced variation in *B. dysenteriæ* by growth in a gum tragacanth abscess in a guinea pig. Agglutinable and inagglutinable strains could be picked according to the different colonies observed after plating the material from the abscess. Ecker (1923) was able to induce similar changes in the Rawlins strain of *B. typhosus* by the injection of this organism into a sterile gum tragacanth abscess in a guinea pig. Simonds (1915) by growing *B. typhosus* in dextrose broth over long periods of time was able to produce a variant of *B. typhosus* which had greatly enhanced agglutinogenic powers.

Summarizing my results in light of the foregoing review, the following facts are apparent: Strains Nos. 12, 21 and 40 were typical in their serological reactions but were irregular in xylose fermentation. They fermented xylose slowly and could not be trained to give rapid fermentation although they produced daughter colonies on xylose plates. Strain No. 5 did not ferment xylose but did ferment rhamnose. It also differed from typical strains of *B. typhosus* in its dextrose metabolism. It was not agglutinated by the patient's serum nor by antityphoid serum. It does not, however, differ any more

radically from typical strains of *B. typhosus* than do those reported by Ecker (1923), Blankenhorn (1923), Berridge (1920) and others. Krumwiede,* in spite of his belief in the stability of antigenic properties falls back on rhamnose fermentation and classes this organism as a paratyphoid strain because of its fermentation of rhamnose. No. 5 was an organism which had no apparent antigenic relation with any other organism of the colon-typhoid group. It had the property of attacking dextrose and proteins simultaneously. In this respect it resembled the colon, para-colon and dysentery organisms. Its resemblances to *B. typhosus* have been summarized above. In its action on rhamnose and xylose, it resembled the paratyphoid group. Since it failed to conform to either the cultural or serological criteria but was irregular in both, it would seem wiser to consider it as an unidentified paralike organism which does not produce gas.

GENERAL DISCUSSION.

The question of the criteria to be used in the identification of typhoid-like organisms and the homogeneity of the *B. typhosus* group has been raised by the work reported here.

The classification of bacteria offers peculiar difficulties since morphological variations are so slight that physiological and antigenic characters must be used. Winslow (1906*a*) points out that in unicellular organisms acquired characters are propably inherited to a higher degree than with other forms, and therefore they are more influenced by environmental conditions. Four methods for the classification of bacteria may be used: First, morphological; second, cultural; third, antigenic; fourth, a combination of the preceding. It is fairly well accepted now that a purely morphological classification like that of Migula is entirely insufficient. Chester (1909), Andrews and Horder (1906), Winslow (1906), and, more recently, Bergey (1923) in his manual have used the cultural method of classification or a combination of cultural and morphological methods. Krumwiede (1918) and others consider the antigenic properties of bacterial protoplasm as much more stable than the physiological reactions, and would therefore place more confidence in the antigenic characters for classification and identification. Jordan (1920) recognizing the correlation between antigenic and cultural characters, would use both for identification.

A survey of the literature bearing on the cultural and antigenic

* Information given in a letter from Dr. Krumwiede after he had examined this organism.

characters used in the classification of the different groups of organisms reveals the fact that homogeneity for any one group is the exception rather than the rule. Streptococci have been classified by fermentation reactions by Gordon (1905), Andrews and Horder (1906), Holman (1916), Blake (1917), and others. The concensus of opinion seems to be that the fermentation of certain carbohydrates is remarkably constant. The antigenic classification of the streptococci has proved difficult if not impossible because of their antigenic heterogeneity. *Streptococcus scarlatinæ* may be an exception in that it is apparently quite homogeneous in its antigenic properties as shown by the work of Dochez (1924) and Dick (1924). With only a few exceptions it has been impossible to correlate fermentation and antigenic characters among the streptococci. It is generally accepted now that the classifications such as Brown's (1919), Blake's (1917), and Holman's (1916), based entirely on physiological reactions, offer the best solution of the problem.

Pneumococci exhibit quite constant physiological reactions as a group, but the work of Dochez (1913), Gillispie (1916), Neufeld (1910), and others, have demonstrated distinct and constant antigenic differences within this group.

Meningococci have been divided into at least four groups which are not as well defined as those of pneumococci.

Torrey and Buchell (1922) have shown that gonococci cannot be divided into clear-cut immunological types, but they suggest a classification under three headings: (*a*) Regular; (*b*) intermediate; and (*c*) irregular strains. They comment on the marked tendency to antigenic lability on the part of the gonococcus and believe that instability of antigenic constitution is a characteristic of this group.

B. diphtheriæ have been cited as being so homogenous as to constitute a true genus. Recently, however, antigenic differences have been demonstrated by agglutination and complement-fixation reactions which subdivide this group into at least two groups. These antigenic differences are not correlated with cultural differences, and it is interesting that there is slight if any correlation between the antigenic differences and toxin production. That is to say, antitoxin prepared from one strain will neutralize toxin from the other strains. There seems to be a difference here between physiological, antigenic and functional characters of the organism.

Clear cut antigenic differences are present among stains of *B. dysenteriæ* and in this case cultural and antigenic differences are closely correlated.

The paratyphoid group has been peculiarly difficult to classify because of the lack of correlation between cultural and agglutination reactions. Sherwood, Downs and McNaught (1920) have observed antigenic differences in organisms resembling *B. enteritidis*. Zinsser (1923) remarked that we should be slow to assume that either agglutination or culture similarity are to be regarded as definitely signifying biological relationship.

Jordan (1918) emphasizes the fact that variations in both fermentation and agglutination are common in the paratyphoid-enteritidis group. The most permanent characters of the group seem to be the fermentation of rhamnose by *B. paratyphosus*, but not by *B. typhosus;* the fermentation of xylose by *B. typhosus*, but not by *B. paratyphosus A*. He considers the above reactions more important than the agglutination reactions.

B. typhosus has long been considered the prototype of an organism in which the protoplasm is antigenically homogeneous. If such is the case, it seems to offer an exception to the general biological rule as brought out by the foregoing survey.

It is also difficult to dismiss as unimportant the numerous reports of definite antigenic differences. It is true that a careful examination of these reports and of my results, as shown in Part I, shows that the differences are apt to be quantitative rather than qualitative. An organism which agglutinates weakly or not at all with antityphoid serum may absorb agglutinins to an appreciable extent or it may produce an antiserum which agglutinates typical strains of *B. typhosus* in high dilution. That it is impossible to use the agglutination or even the absorption reaction as an absolute criterion for identification of *B. typhosus* strains, is indicated by the interagglutination and absorption reactions between *B. typhosus* and *B. pullorum* and *B. sanguinarium*. Here there is a marked antigenic similarity and somwhat less marked cultural similarity between organisms entirely unrelated as to source and pathogenicity.

The occurrence of agglutinative and cultural irregularities in certain epidemics of typhoid fever point to a possible sanitary significance of different strains of *B. typhosus*. Many suggestions have been offered as to the advisability of using more than one strain in typhoid vaccines. My results point to the occurrence of such distinct and constant antigenic differences between certain strains of *B. typhosus* that the use of a polyvalent vaccine would be advisable.

It would seem wiser in view of the foregoing reports and the re-

sults of my experiments to use a combination of cultural and antigenic characters for identification of organisms.

The utility of any reaction for identification purposes involves the following factors: First, the constancy of the reaction under stated conditions; second, spontaneous variations in the reactions; third, induced variations.

The apparent constancy of cultural reactions depends to a marked degree on the medium used, the amount and age of inoculum, the time and temperature at which the experiment is carried out.

My results show that the buffer content of the medium exerts a profound effect on sugar metabolism. With a vigorously growing organism it would be entirely possible to use up all the fermentable carbohydrate present and show an alkaline reaction in twenty-four hours if insufficient carbohydrate was used.

The effect of the presence of a fermentable carbohydrate in protein metabolism has been clearly shown by Kendall (1916), Sears (1917), DeBord (1923), and others. In using cultural reactions for identification, it is necessary, therefore, to standardize the culture medium and the tests used to indicate the various reactions taking place.

It seems possible that the constancy of antigenic properties might also be influenced by the culture medium on which the organisms are grown. Simonds (1915) has shown that continuous growth in the presence of dextrose influenced the antigenic properties of a certain strain of *B. typhosus*.

Salts, hydrogen-ions and colloids in the suspension of organisms used and in the antiserum have a great influence on the demonstration of antibody reaction in the agglutination and absorption reactions as well as in other immunological reactions.

Here again a standardization of these variable factors would be desirable and would make it possible to compare the results obtained by different authors.

The occurrence of spontaneous and induced variations in bacteria in both cultural and antigenic reaction has frequently been noted Berridge and Glynn (1920), Broadhurst (1915), and others, have noted the spontaneous acquisition or loss of fermentative characters in a number of organisms. The spontaneous acquisition of agglutinability by strains which are inagglutinable when first isolated is well known. Toyoda (1922), Mock (1919), Benians (1920), and others, have reported on sponctaneous changes in agglutinability in cultures of *B. typhosus*.

Enhanced fermentation reactions have been reported by Twort (1907), Penfoed (1912), Muller (1919), D'Aunoy (1913), and many others, by the continuous growth of an organism in the presence of carbohydrates. My results on this point have been entirely negative. Dawson (1919) has been able to induce variation in both cultural and antigenic properties in *B. coli* by growth on various media. Bergstrand (1923), Berridge and Glynn (1920), and Jordan (1926), have reported the occurrence of antigenic variability in organisms picked from different colonies of pure cultures of *B. coli*, *B. dysenteriæ* and *B. paratyphosus B.*

Ecker (1923), Blankenhorn (1923), Gay (1913), Meyer (1921) and others, have obtained changes in antigenic properties in *B. typhosus* by animal inoculation.

It must be recognized that it is only occasionally that the spontaneous and induced variations noted above have any practical bearing on the identification of organisms. However, whenever an organism is to be identified the possibilities of such irregularities must be considered. There seems to be meager evidence as to the stability of these variations. The antigenic and cultural variations reported by me have remained unchanged for three years.

In summing up the criteria to be used in the identification of organisms, the following factors should be considered.

1. Physiological reactions, including (*a*) morphological and staining reactions, (*b*) cultural reactions, both fermentation of carbohydrates and protein utilization.

2. Antigenic reactions, including agglutination and absorption tests, and in some cases complement fixation, toxin production and pathogenicity.

3. A correlation of the above factors and a consideration of the significance of any variations noted.

In the results reported here I found the strains of *B. typhosus* used to be homogeneous in regard to the cultural reactions with the exception of three strains which fermented xylose slowly and one strain which gave a rapid return to alkaline in litmus milk. These strains were much less homogeneous antigenically than they were culturally. It would seem from my results and the result of others that *B. typhosus* is less homogeneous in its antigenic properties than is commonly supposed. It, therefore, conforms to what seems to be a general biological law among bacteria, namely, that organisms within the same group exhibit antigenic and cultural variations, and that these variations are not necessarily correlated.

GENERAL SUMMARY AND CONCLUSIONS.

The following conclusions were drawn from the study of thirty-one strains of *B. typhosus*. One unidentified organism, No. 5, was considered separately:

1. Qualitative cultural reactions were constant in glucose, mannite, maltose, sucrose, lactose and rhamnose. Irregularities in dextrin and salicin fermentation and litmus milk were noted. Several strains fermented xylose slowly. These irregularities could not be correlated with the age of the cultures nor the source. No variation occurred in indol reaction, gelatin liquefaction, nor lead-acetate reaction.

2. Antigenic irregularities as demonstrated by agglutination, absorption and Widal tests occurred in these strains of *B. typhosus*. These irregularities were constant, but were quantitative rather than qualitative. The strains used may be divided into three groups on the basis of these reactions.

3. These antigenic irregularities were sufficiently constant to make advisable the use of several strains for Widals and also possibly for typhoid vaccines.

4. The growth range of *B. typhosus* was found to be pH 5.0 to pH 9.0, the optimum range, from pH 6.0 to pH 7.5.

5. There was a slight increase in titratable acidity in broth to which no carbohydrate had been added due to the presence of muscle sugar. The hydrogen-ion concentration and titratable acid after twenty-four hours became increasingly alkaline.

6. The metabolism of these strains of *B. typhosus* in broth containing various carbohydrates was remarkably constant. The final hydrogen-ion concentration reached was characteristic for each carbohydrate with the strains used.

7. The final hydrogen-ion concentration was usually reached in twenty-four hours.

8. A definite amount of dextrose was necessary to enable the organisms to reach their final hydrogen-ion concentration, and the amounts varied with the buffer content of the medium.

9. The initial hydrogen-ion concentration had little effect on the metabolism of the organisms, provided it was within the optimum range of growth.

10. The viability of the organisms was determined by the free hydrogen-ions produced by fermentation and by the kind of carbohydrate fermented.

11. Slight but distinct alkaline reversion occurred after the final hydrogen-ion concentration was reached. The lower hydrogen-ion concentrations given in less available carbohydrates was correlated with a greater degree of alkaline reversion. This slight alkaline reversion did not signify complete utilization of the carbohydrate.

12. The amount of dextrose used and the acid formed was constant for all the strains under stated conditions, that is to say, the composition of the medium determined the amount of dextrose used and the accumulation of acids. The presence of buffers stimulated the metabolism of dextrose by the bacteria much more than it stimulated growth.

13. The amount of dextrose used and titratable acid paralleled each other closely for seventy-two hours. The change in hydrogen-ion concentration lagged behind the accumulation of acids and the utilization of dextrose so that it would be impossible to use the change in hydrogen-ion concentration as an accurate index of the amount of dextrose used, the change in titratable acid or growth rates.

14. Titratable acidity offered a more complete indication of the amount of dextrose used than did the change in hydrogen-ion concentration.

15. The period of maximum dextrose utilization per organism preceded the period of maximum reproduction. The period of logarithmic increase coincided, however, with the period of the greatest total decrease in dextrose.

16. It was impossible to correlate the antigenic differences noted in Part I with any peculiarities in cultural or metabolic reactions.

17. An unidentified organism was described—No. 5. It was inagglutinable in all sera used. It resembled *B. typhosus* in its cultural reactions except that it did not ferment xylose and did ferment rhamnose. The metabolism of this organism in dextrose resembled that of *B. dysentery* or *B. coli* in that simultaneous acid and alkaline fermentation seemed to occur. It seemed to be a new organism which might ultimately be classified as a member of the dysenteriæ group or as a non-gas-producing paratyphoid.

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Studies on *Bacillus pyocyaneus*.

NOBLE P. SHERWOOD, T. L. JOHNSON, AND IDA RADOTINCKY,
Department of Bacteriology.

SINCE Gessard (1882) first isolated *B. pyocyaneus* in pure culture, an extensive literature on this organism has accumulated. The investigations have been done almost exclusively along four lines; *i. e.*, its proteolytic power, pathogenicity, antagonistic action for certain bacteria, and the pigments produced by it. Jordan (1899 *a*, 1903) has made the most notable contributions in the latter field, and has also shown that its hemolytic property is due to the alkali produced. The work of Wassermann (1896) on toxin production would apparently need further investigation. Pribram and Pauley (1915) reported upon the agglutination reactions within the fluorescent group of bacteria. They noted a strong antigenic relationship between *B. pyocyaneus* and *B. fluorescens liquefaciens*. While its ability to produce proteolytic and reducing enzymes has been extensively studied, very little has been done in regard to its ability to ferment carbohydrates, higher alcohols, etc. Flügge (1896) mentions its ability to ferment glycerol. Jordan (1899 *a*) (1899 *b*) states that *B. pyocyaneus* does not produce gas in dextrose broth fermentation tubes. He noted fermentation of dextrose by other fluorescent organisms. Fuller (1899), on the other hand, reported gas production, while McFarland (1919) states that *B. pyocyaneus* does not ferment carbohydrates. De Bord (1923) has recently reported that the rate of production of amino nitrogen in cultures of *B. coli*, *Ps. pyocyanea*, *B. subtilis*, *Cl. botulinum*, and *Cl. sporogenes* in peptone media is accelerated when glucose is present. He further states that *Ps. pyocyaneus* destroys glucose without markedly increasing the hydrogen-ion concentration and concludes that pH determination is fallible as an index of sugar destruction.

Clawson and Young (1913), working in this laboratory, reported cyanide production by *B. pyocyaneus*. Patty (1921) continued this work by studying the optimum conditions for cyanide production and comparing the quantities produced by nine strains. The present paper is the third in this series. The number of strains has been increased to twenty-two. The experimental work herein embodied has been carried out with the hope of throwing light upon the following questions:

1. How many, if any, of the twenty-two strains ferment dextrose and lactose as judged by growing them in 1 per cent sugar meat infusion broth and Hiss serum water media, using litmus as an indicator?

2. Would quantitative sugar determinations on cultures grown in 1 per cent dextrose meat infusion broth indicate sugar utilization by any of the strains giving negative results with litmus as an indicator?

3. What would be the hydrogen-ion concentration developing in such cultures?

4. What would be the reactions of a reasonable number of the strains in mannite, saccharose, arabinose, dulcite, xylose, salicin and rhamnose?

5. What would be the results of comparative studies on their ability to blacken lead acetate agar, produce indol in Dunham's peptone solution and reduce nitrates to nitrites?

6. How do the additional thirteen strains and also a culture of *B. fluorescens liquefaciens* compare as to cyanide production?

7. How do the twenty-two strains compare antigenically as evidenced by the agglutination reaction.

MEDIA AND METHODS.

1. Plain meat extract broth and fresh meat infusion broth, using Witte's peptone, and made according to standard methods, were used as bases for the sugar broths and agar media employed. The customary method for preparing Hiss serum water media was followed. A 1 per cent concentration of the fermentable substance was added. The broth was tubed in 10 cc. amounts in hard glass test tubes and in 150 cc. amounts in pyrex glass Erlenmeyer flasks. One-tenth cc. of a 24-hour meat infusion broth culture was used to inoculate the tubes and 0.5 cc. for inoculating the flasks. Pigment production and reduction of litmus were factors which prevented satisfactory readings to be made for more than a few days except where an adsorbing substance was added.

2. The hydrogen-ion concentrations were determined by the colorimetric method as suggested by Clark and Lubs. To prevent interference of pigment, one gram of sterile animal charcoal was added to each 150 cc. of media in the flasks. This apparently adsorbed the pigment. Control flasks of sterile media were used. The cultures were carried along in triplicate for two-week periods. This work was rechecked, thus giving six sets of determinations for each organism. The buffer solutions used were checked by means of a potentiometer.

3. Quantitative sugar determinations were done on a number of strains using Benedict's method.

4. Lead acetate agar was prepared as recommended by Jordan (1917).

5. Indol was tested for by the vanillin reaction (Nelson, 1916).

6. Cyanide determinations were made as previously described by Patty (1921).

7. For agglutination work the macroscopic method was used.

SOURCES OF ORGANISMS USED.

The history of the organisms studied is given in Table I.

TABLE I.

Strain No.	*Source.*
68	American Museum.
174	University of Chicago and Rush Medical College.
175	University of Chicago and Rush Medical College.
176	University of Chicago and Rush Medical College.
177	University of Chicago and Rush Medical College.
178	Gizzard strain.
179	University of Michigan.
180	University of Chicago and Rush Medical College.
184	Stool of influenza patient at Lawrence, Kan.
250	American Museum strain No. 150.
251	American Museum strain No. 151.
252	American Museum strain No. 152.
253	American Museum strain No. 156.
254	American Museum strain No. 206.
255	Dawson strain, Bureau of Animal Industry. American Museum strain No. 102.
256	American Museum strain No. 129 (The Jackson strain).
257	Bullock strain, Lister Institute No. 42 A.
258	Goat strain, Lister Institute No. 254.
259	Fildes strain, Lister Institute No. 150 B.
260	From the stool of an influenza patient at Lawrence, Kan.
261	From the stool of a typhoid fever patient at Lawrence, Kan.
262	From the stool of a typhoid fever patient at Lawrence, Kan.

RESULTS.

The results of the fermentation reactions, growth in lead acetate agar, Dunham's peptone solution and nitrate broth are shown in Table II. This table also contains the results of quantitative cyanide determinations for each strain grown in synthetic broth as described by Patty (1921).

TABLE II.

Strain No.	Results of carbohydrate fermentation as determined by indicators.									Blackening of lead aceate agar.	Indol.	No. 2.	HCN mg. per 100 cc.
	Dex.	Lact.	Man.	Sacch.	Arab.	Dul.	Xyl.	Sal.	Rham.				
68....	—	—	—	+	—	—	—	—	+	—	+	—	.05925
174....	—	—	—	+	—	—	+	+	—	—	+	—	.....
175....	+	—	—	+	—	—	—	+	+	+	+	—	.....
176....	+	—	—	+	—	—	—	—	+	+	+	—	.....
177....	+	—	—	+	—	—	—	+	+	+	+	—	.....
178....	—	—	—	+	—	—	+	+	+	—	+	—	.00953
179....	+	—	—	+	—	—	—	+	—	+	+	—	.....
180....	—	—	—	+	—	—	—	+	+	—	+	—	.....
184 ...	+	—	—	+	—	—	—	—	—	—	+	—	.....
250....	—	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.02706
251....	+	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.00766
252....	+	—	.....	.....	.....	.....	.....	.....	.....	—	+	—	.02041
253....	—	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.01069
254....	—	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.02113
255....	+	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.08010
256....	?	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.01910
257....	+	—	.....	.....	.....	.....	.....	.....	.....	—	+	—	.01832
258....	+	—	.....	.....	.....	.....	.....	.....	.....	—	+	—	.04330
259....	+	+	.....	.....	.....	.....	.....	.....	.....	+	+	+	.01688
260....	+	—	—	+	—	—	—	+	+	—	+	—	.02695
?261....	?	—	.....	.....	.....	.....	.....	.....	.....	—	+	—	.04950
262 ...	+	—	.....	.....	.....	.....	.....	.....	.....	—	+	—	.01550
B. fluorescens liquefaciens.....													.05126

Table III shows a number of hydrogen-ion concentrations for each organism grown in fresh meat infusion dextrose broth having an initial pH of 7.0 and containing 1 gram of animal charcoal to adsorb the pigment.

TABLE III.

STRAIN NO.	Hydrogen-ion concentration in 1% meat infusion dextrose broth expressed in pH.								
	Days.								
	0	1	2	3	4	5	6	7	14
68	7.0	7.0	7.0	7.2	7 6	7.6	7.8	8.0	8.0
174	.....	7.0	7.0	7.2	7.2	7.8	7.8	8.0	8.0
175	.....	7.0	7.0	6.8	6.6	6.6	6.6	6.6	6.6
176	.....	7.0	6.8	6.8	6 8	6.8	6.8	6.8	6.8
177	.....	6.6	6.8	7.0	7.0	7.4	7.6	7.6	7.8
178	.....	7.0	7 0	7 4	7.4	7.4	.....	7.8	7.8
179	.....	7 0	7 0	7.0	7.0	6.8	6.6	6.4	6.4
180	.....	7.2	7 2	7.4	7.4	7.6	7 6	7.8	7.8
184	.....	7.0	6.6	6.6	6.6	6.6	.....	6.6	6.0
250	.....	7 0	7.0	7.6	7.6	7.6	.....	8.0	8 4
251	.....	7.0	.....	6.6	6.6	7.0	.....	7.4	7.6
252	.....	6.4	.....	6.2	6.8	7.0	.....	7.0	7.6
253	.....	7.0	.....	7.0	6.6	7.0	.....	7.2	8.0
254	.....	7.0	.....	7.4	7.4	7.4	.....	7.8	8 0
255	.....	6.4	.....	6.4	6.8	7.0	.....	7.2	7.2
256	.....	7.0	.....	6.6	6.6	6.8	.....	7 0	7.6
257	.....	6.6	.....	6.8	6.8	7.0	.....	7.0	7.6
258	.....	7.0	.....	7.0	5.6	5.8	.....	6.0	6 8
259	.....	5.8	.....	5.4	5.4	5.4	.....	5.2	5.0
260	.....	7.0	.....	6.8	6.4	6.4	.....	6.2	7 0
261	.....	6.8	.....	7.2	7.4	7.6	.....	7.8	8 0
262	.....	6.6	.....	6 6	6.6	6.6	.....	7 0	7 8

Table IV gives the results of studies on seven strains of *B. pyocyaneus* to ascertain their ability to utilize dextrose. Three of these strains had given negative reactions in dextrose broth using litmus as an indicator, while the other four strains had shown an acid reaction. The reactions to litmus and the hydrogen-ion concentration developing in a neutral dextrose charcoal broth are herein compared with the percentage of sugar utilized daily for four days.

TABLE IV.

STRAIN NO.	Reaction in litmus dextrose meat infusion broth.	PH in 1% meat infusion dextrose charcoal broth.						Dextrose concentration of broth expressed in grams per 100 cc.				
		Days.						Days.				
		0	1	3	5	7	14	0	1	2	3	4
68	—	7.0	7.0	7.0	7.6	8.0	8.0	1.14	1.10	0.92	0.67	0.67
178	—	7.0	7.0	7.4	7.4	7.8	7.8	1.14	1.14	1.12	1.06	1.06
250	—	7.0	7.0	7.6	7.6	8.0	8.0	1.14	1.14	1.05	0.72	0.51
184	+	7.0	6.6	6.6	6.6	6.6	6.0	1.14	0.97	0.77	0.72	0.69
252	+	7.0	6.4	6.2	7.0	7.0	7.6	1.14	1.13	1.02	0.59	0.54
259	+	7.0	5.8	5.4	5.4	5.2	5.0	1.14	1.03	0.91	0.83	0.71
260	+	7.0	7.0	6.8	6.4	6.4	7.0	1.14	0.88	0.78	0.69	0.61

DISCUSSION OF TABLES II, III, IV.

An inspection of Table II indicates that fifteen out of the twenty-two strains ferment dextrose, and one strain, the Fildes strain, obtained from the Lister Institute, fermented both dextrose and lactose. These results are confirmed by the hydrogen-ion concentrations tabulated in Table III. The data in Table IV suggest that the apparently negative strains did utilize dextrose, but that the alkali produced kept pace with acid production, and finally alkali production predominated. This is of interest, since it apparently throws more light upon the principle of the interference of fermentation with proteolysis and is in keeping with what one would expect from the standpoint of physiology. The principle that the presence of a fermentable carbohydrate in a culture would in general result in proteolysis being held in abeyance until the concentration of carbohydrate had been reduced to an exceedingly small amount has been studied extensively by Kendall (1921). Peckham (1897) announced this phenomenon for the colon group, and mentions that Pere had previously called attention to it. Our results suggest that, given the proper hydrogen-ion concentration, both processes may be initiated.

Subsequent results depend largely upon the development of an optimum hydrogen-ion concentration for either fermentation or proteolysis. Apparently, sugar was utilized at pH 7.6 which is not an incompatible reaction for proteolysis. These results, as well as those of DeBord (1923), and Downs (1924) of this laboratory, also emphasize the limitations of the indicator methods in deciding upon the ability of an organism to ferment a carbohydrate. Mannite, arabinose, and dulcite were apparently negative, while saccharose was uniformly fermented by ten of the strains studied. The results in xylose, salicin and rhamnose were variable.

There was great uniformity in the production of indol, but only half of the twenty-two strains blackened lead acetate agar. Only one reduced nitrates to nitrites. Jordan (1899*a*) noted that his cultures of *B. pyocyaneus* produced indol, but that the fluorescent organisms gave variable results. He also noted that nitrates were strongly reduced by the Rush strain of *B. pyocyaneus*, slightly reduced by *B. pyocyaneus alpha*, and not reduced by five other strains. Tanner (1917) studied ninety-seven strains of fluorescent bacteria and one strain of *B. pyocyaneus* to ascertain whether any or all of these organisms produced hydrogen sulfid. He found that the majority of the fluorescent bacteria produced it, but that the one culture of *B. pyocyaneus* failed to do so. Sasaki and Otsuka (1912) noted that one strain of *B. pyocyaneus* did not produce hydrogen sulfid.

In regard to cyanide production, our results were quantitatively less than reported by Patty (1921). This was probably due to environmental factors in the culture media, as the same methods were used and repeated checks made. The production of cyanide by *B. fluorescens liquefaciens* (American Museum strain) is another characteristic in common with *B. pyocyaneus*.

ANTIGENIC RELATIONSHIP.

For this work adult rabbits were immunized against strains 68, 174, 175, 176, 177, 178, 179, 180, 184 and 260 respectively. Two rabbits were inoculated for each strain. Each animal received ten subcutaneous inoculations at weekly intervals and several small doses of live cultures intravenously. The results were disappointing. None of the titres were high, but varied from zero to 1-5000. The results of cross agglutination were irregular.

SUMMARY AND CONCLUSIONS.

The results of the comparative study of the carbohydrate and protein metabolism and of the antigenic relationship of twenty-two strains of *Bacillus pyocyaneus* maye be summarized as follows:

1. Practically all strains of *B. pyocyaneus* have the ability to utilize dextrose although alkaline products may obscure this phenomenon. They were consistently negative in mannite, arabinose and dulcite, and positive in saccharose.

2. Our results as well as those of DeBord (1923) and Downs (1924) emphasize the limitations of hydrogen-ion determinations as an indication of sugar utilization.

3. The Fildes strain was the only one of the twenty-two strains studied that showed acid production in litmus lactose broth. It also produced the maximum hydrogen-ion concentration in fresh meat infusion dextrose broth, reaching a pH of 5.0 in two weeks. Six of the twenty-two strains showed an acid reaction after two weeks. The remaining sixteen strains developed pH values ranging from 7.0 to 8.0 at the end of fourteen days. The initial reaction was pH 7.0. These results might not hold if the initial hydrogen-ion concentration were *decidedly* different.

4. Animal charcoal added to dextrose broth apparently adsorbs the pigments.

5. All strains produced indol.

6. Half of the twenty-two strains blackened lead acetate.

7. Only one of the twenty-two strains reduced nitrates.

8. All strains produced HCN, as did also a strain of *B. fluorescens liquefaciens*.

9. Efforts to produce high titred immune sera were not very successful; a titer of 1-5000 was the maximum obtained. Cross agglutination experiments gave irregular results.

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The Genus *Erythroneura* North of Mexico.

(Homoptera, Cicadellidæ.)

WILLIAM ROBINSON, Department of Entomology.

THE genus *Erythroneura*, which contains the grape leaf hoppers, is well known to both economic and systematic workers. It has been called the most intricate genus in the entire family; it is certainly one of the most interesting. Much has been written about the forms which attack the grape, and their life history and control measures have been well studied. However, strange as it may seem in the case of important economic insects, a good deal of uncertainty and confusion has existed as to the species. In many publications the grape leaf hoppers have been erroneously named and classified, and several distinct species have been called varieties of *comes*. Thus our commonest grape leaf hopper has been credited altogether with as many as twenty varieties. In discussing with economic workers the taxonomic position of the various forms which attack the grape, I have been able only with difficulty to get them to express an opinion at all as to species. I have therefore undertaken to gather together all the known American species, north of Mexico, and to study them from every possible standpoint, as explained later.

The species are numerous, widely distributed and very abundant in individuals. Color pattern is one of their most striking characteristics, comprising a wide range of color, and assuming, in some species, a pattern approaching the bizarre—black spots within red rings upon a white background; zigzag streaks of yellow, red or brown upon white; dots or dashes of red upon ivory; wide cross-bands of velvety black upon white; a large central area of maroon on a background of cream; neat patterns in pink, rose or vermilion upon white or cream; or the color may be brown, red or black with white spots.

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Being of small size, however, they are easily overlooked or neglected. Nevertheless they are far from insignificant in pattern and in their ability to do injury.

In the absence of better external characters, these color patterns have been used to distinguish species. In certain instances this was simple; but in many cases, when using only these characters, it was difficult and almost impossible to make accurate determinations. This is due partly to the variations which were supposed to exist within the species, and to the merging of the color pattern of one species into that of another, thus forming complexes which were exceedingly confusing. Under such circumstances it was impossible for anyone to make an accurate determination of many of the species. In McAtee's revision,[1] the author separated the genus into groups based on characters presented by wing venation. This, despite a certain amount of variation, was a considerable improvement over the old system. However, it was still impossible to say definitely whether forms having slight differences in color pattern were distinct species or merely varieties.

The present work embodies a study of the inner male genitalia, as well as of wing venation and of color pattern, and it is believed that the characters presented by the genitalia, which are practically constant within the species and which have outstanding specific characteristics, will add much to the definiteness of classification. It is interesting to note that in most cases there is a marked correlation of these three factors. For instance, in the *comes* group the vein cubitus turns abruptly toward the inner margin of the wing and thus forms a square base for cell M_4. (Plate I.) All species of the *comes* group have this type of venation. Correlated with this is the occurrence in every case of a black dot or cloud in the apex of cell R_3 and the base of cell M_4. Also the pygofer hooks of the inner male genitalia are bent to form more or less U-shaped processes (Plate I), and are of a shape not found in any species outside this group.

In the *maculata* group cubitus turns forward and forms an oblique base for cell M_4. (Plate I.) These species have a black dot in the base of cell M_4, the apex of cell R_3 is clear; while the pygofer hooks consist invariably of long, spearlike processes.

In the *Obliqua* group the cubitus turns forward, but the base of cell M_4 is made up of the m-cu cross-vein along with the bent por-

1. Key to the Nearctic Species and Varieties of *Erythroneura:* Trans. of the Am. Ent. Soc., XLVI, 267-322; 1920.

tion of cubitus, and these two parts are joined together to form a more or less smoothly curving line. In the color pattern may be found oblique vittæ upon the tegmina or an inverted "V" mark on the vertex and pronotum, or both of these characters. The pygofer hooks are short and thick, with an apical point and a ventral stub-like process.

All the North American species which have been described up to the present time, together with a large number of variations, have been studied. Thirty new species have been added to the list and twelve varieties have been given specific rank, based upon the study of their inner male genitalia. The genus now contains sixty-two[2] species and twenty-six varieties.

Acknowledgments are due to Dr. P. B. Lawson, at whose suggestion this work was undertaken and who was ever ready with assistance and advice as the work progressed; to Prof. R. H. Beamer, for collecting a large amount of Kansas material; to Mr. W. L. McAtee, for his kindness in supplying the writer with many species for study and dissection; also to Mr. E. P. Van Duzee, Prof. C. P. Gillette, Dr. D. M. DeLong and Mr. L. Stearns, for sending in material for examination. I have made extensive use of McAtee's original keys to groups and species.

Some notes on the bionomics of the genus, based mostly on personal observations over a period of several years, are here included.

LIFE HISTORY.

The members of this genus pass the winter as adults. In the Southern states they remain more or less active throughout this period. In Canada and in the Northern and Middle states the adults go into hibernation upon the approach of cold weather, chiefly under the protection of fallen leaves, but they may also be found within clumps of dead grass, under loose bark of trees, under fallen logs, and in decaying tree stumps. A favorite situation seems to be in the deep layers of dead leaves which have been lodged by the wind at the base of trees, stumps and bushes.

An excellent opportunity to study the adults while in their hibernating quarters occurred in Kansas during the winter of 1923-'24. The ground was rarely covered with snow and observations could be made at any time. A study of the effect of rapid changes of tem-

2. Since writing the above, four new species and one new variety have been described by E. P. VanDuzee: Proc. Cal. Acad. Sc., 4th series, vol. XIII, pp. 231-236; 1924; and three new species and four new varieties have been described by W. L. McAtee, Division of Natural History Survey (Illinois), vol. XV, art. II; 1924.

perature upon the adults was possible. Sharp freezing spells of short duration followed by warm, sunny days were of frequent occurrence. At one time the temperature went down to eighteen below zero and within forty-eight hours had risen to forty degrees above. A large percentage of insects of other families and orders which passed the winter in the adult stage were found dead in the spring; but *Erythroneura* wintered well, not more than one in one thousand being found dead.

The adults emerge from their winter quarters and commence to feed in the late spring, the sunshine of early spring not being sufficient to warm through the layers of leaves. In the case of many species, among them the grape leaf hoppers, the emergence of adults in the spring takes place before their host plants have begun to come into leaf, and in the meantime they feed upon a great variety of available plant foliage. This is true of the species in general; and the fact that species are taken on any given plant in the spring and in the late autumn does not imply that that is its host plant.

It is necessary to make some distinction between host plants and temporary food plants. The former are those within which the females lay their eggs and upon which the nymphs as well as the adults feed; the latter are those upon which the nymphs are never found and only the hibernating adults feed in the absence of their host plants. I have taken the grape leaf hoppers on as many as eighty different species of food plants, but have found them breeding on only the grape or allied plants. The injury to the temporary food plants is usually negligible.

Copulation may take place upon the spring food plants and upon the host plants, to the latter of which both males and females proceed shortly after the earliest appearance of the foliage. The eggs are minute, elongate, whitish, and are laid within the tissues of the leaf. They may be scattered or placed side by side in a row, depending upon the species. The nymphs pass through five instars, in common with other members of this family.

By the time the nymphs have reached maturity practically all the overwintering forms have died. The new brood mate, and in the Southern states may produce a complete second brood, while in the North only a partial second brood is produced. In the autumn the host plants may die before cold weather forces the adults into hibernation; and until cold weather appears the hoppers will go to and feed upon a number of available food plants just as in the spring.

In some species and varieties both yellow and red forms occur. The reddish colors develop in the late summer and are retained throughout hibernation. In the following spring after the adults have begun to feed upon the newly formed foliage, the reddish vittæ may turn yellow.

NATURE OF INJURY.

Both nymphs and adults feed upon the under side of the leaves. They extract juices from the foliage and take along at the same time chlorophyl from the cells. At every feeding place a small white spot appears which is made up of several minute points. Each point represents a feeding puncture and indicates that the hopper inserts and withdraws its mouthparts several times at each feeding place. These spots increase in number and coalesce, making pallid areas which may in time cover the whole surface of the leaf. The puncturing of the epidermis in innumerable places eventually causes a drying up of the leaf tissue, and in the case of serious injury it becomes brown, dry and functionless. This injury to the foliage has a direct effect upon the fruit. In badly infested vineyards there is a marked decrease in the sugar and an increase in the acid content of the fruit; and a decided lowering of the total yield takes place. Also it is not uncommon in the case of red varieties of grapes for the crop to fail to ripen sufficiently to be picked.

The cultivated grape is by far the most important plant attacked by the members of this genus. The following is a list of species and varieties which breed upon the grape:

E. comes Say.
E. comes var. *octonotata* Walsh.
E. comes var. *compta* McAtee.
E. coloradensis Gillette.
E. vitifex Fitch.
E. rubra Gillette.
E. portea Robinson.
E. vulnerata Fitch.
E. niger Gillette.
E. ziczac Walsh.
E. vitis Harris.
E. tricincta Fitch.
E. illinoiensis Gillette.
E. lacta Robinson.
E. elegans McAtee.
E. maculata Gillette.
E. amanda McAtee.
E. obliqua Say.
E. dorsalis Gillette.
E. crevecoeuri Gillette.

Of these, *ziczac* and *elegans* also breed extensively upon *Ampelopsis* spp. which are of the same family as the grape; and *obliqua* has been found breeding upon both wild and cultivated plums.

CONTROL.

Control may be readily effected by the use of nicotine-sulphate, such as "Black Leaf 40," applied at the rate of 1-1200 or 1-1600. The spray should be applied to the first brood nymphs before the

most advanced have reached the fifth instar; and, as the nymphs feed upon the under surface of the foliage, especial care should be taken to drench thoroughly the under surface of all the leaves. A short rod with an angle nozzle should be used; the ordinary long rod is very inconvenient to use in a vineyard. The disc in the nozzles should have a medium-sized hole in order to give a dense but not too fine spray; and the pressure should be from 150 to 200 pounds. Thorough drenching is essential, and from 150 to 250 gallons per acre, depending upon the amount of foliage, should be used.

A long series of experiments[3] has shown that this spray will also destroy the eggs beneath the epidermis, so that it is possible to get excellent control by spraying at the time mentioned above and not waiting until all the eggs have hatched. Delaying the treatment will allow many of the nymphs to mature, and these are not readily killed with a spray.

In the South good use is often made of the sticky trap or hopper-dozer, which is passed over the vines and which catches the adults as they fly upward.

It is a good practice in the early spring to burn over fallen leaves and dead grass along ditches, roadsides and neglected places, in the neighborhood of the vineyard. This may destroy large numbers of hoppers since it is in such places that the adults pass the winter.

COLLECTING.

Winter is a good time for collecting. The favorite place of the adults appears to be, as previously mentioned, among layers of fallen leaves, especially where lodged by the wind against stumps and the bases of trees and shrubs. There the hibernating adults gather in large numbers from their scattered food plants and may be very easily obtained.

There are two or three satisfactory methods of capturing the adults in winter. One is to lift the dried leaves and pick up the dormant insects by the legs with a pair of fine tweezers and put them into a cyanide bottle. Another way is to place the layers of leaves in a fine sieve and shake them over a large funnel into a box or bottle. If this is done on cold days the hoppers will be dormant and fall readily into the container.

In the spring countless numbers of hoppers can be found feeding

3. Susceptibility of Grape Leaf Hopper Eggs to Nicotine: Agricultural Gazette of Canada, vol. X, No. 3; 1923.

upon the foliage of various bushes and low-growing plants in the woodlands. Species of many kinds feed together for several days between the time of emergence from hibernation and the general dispersal to their respective host plants; and it is during this period that the most profitable collecting can be done.

A strong sweeping net of fine close weave attached to a short handle is generally found most satisfactory. Since the hoppers are exceedingly active and readily take flight and dart out of the net, very many may be lost in taking individuals from the net in a small bottle. A better way is to use a net with sides that taper to a point in which the captured adults will fall and remain while the net is in motion. When a number have been caught it is very easy to transfer them altogether by inserting a bottle with a large mouth as far as it will go and shaking the hoppers in. Practically none will escape if handled in that way.

MOUNTING.

Mounting on a celluloid point with shellac or Canada balsam is sometimes practiced, also on a white card point with shellac or liquid glue. The latter method is probably the most satisfactory. The main consideration is that the specimen be securely fastened to the point. A quick way to mount is to place about one inch apart on a piece of cork several pins bearing their card points which should be turned to the left. The specimen is best picked up by moistening on the tongue a bent needle point mounted in a wooden handle, and touching it to the back of the insect to be mounted. A small drop of liquid glue should then be applied to the point by the left hand and the specimen mounted near the tip. I have found that these insects are much less likely to come off if the glue is applied in each case just before the mounting.

CLASSIFICATION.

In this classification the venation of the fore wing is used to separate the genus into groups only, no specific venational characters having been found. In naming the veins, the Comstock-Needham system is used.

The genus *Erythroneura* is separated from the genus *Typhlocyba* by R_{4+5} separating from R_{2+3} and meeting media at right angles, so that cell R_3 has a distinct base instead of being stalked, and by vein M_{3+4} not proceeding straight to tip of wing but curving to meet the inner margin. (Plate I.)

INNER MALE GENITALIA.

The outer male genitalia have not been found to show specific characters, but the inner male genitalia have outstanding differences and provide abundant specific characters which are practically constant for the species as shown by the examination of a long series of individuals.

The parts comprising both outer and inner male genitalia are shown in Plate I. The inner genitalia include a pair of pygofer hooks, one of which is partly imbedded in the upper surface of each pygofer near the anal tube; a pair of styles or claspers whose toothed or apical ends project outside the genital chamber; the œdagus; and the connective which unites the œdagus to the styles. Both dorsal and lateral views of these members are shown and also the position which the inner genitalia occupy within the chamber.

TECHNIQUE FOR PREPARING GENITALIA FOR STUDY.

The most satisfactory method of procedure in preparing specimens for study has been found to be as follows: A number of small vials are numbered and partly filled with a 10 per cent solution of caustic potash. The specimens to be examined are left mounted on their card points and the posterior part of the abdomen is taken off under the binocular and transferred on the moistened tip of a needle to a vial. The specimen on the card point is then given a number corresponding to the one on the bottle. The abdomen is allowed to remain in the cold solution for twenty-four to thirty-six hours. If quicker results are required, heating the solution, but not boiling it, will cut down the time to about half an hour.

A minute pin inserted into a thin wooden handle makes an excellent dissecting point. A device to reach into the bottle and lift out the abdomen without crushing it is made by bending a point of a fine pin to form a minute loop. The stem is then bent at an angle and inserted into a wooden handle.

The tip of the abdomen, after being thoroughly cleared, is transferred to a flat-bottomed watch glass and examined under water with the highest power of the binocular. The abdomen is placed on its side and with strong transmitted light the pygofer hooks can be seen. A drawing of the lateral aspect of the hooks is then made.

In order to get accurate drawings the use of a micrometer disc in the ocular and paper ruled with squares to correspond to the squares in this scale is recommended. The squares on the paper should

measure at least three inches and two of these squares will be enough.

When a lateral view of the pygofer hooks is drawn, the inner genitalia should be very carefully dissected out. The greatest care is necessary to avoid mutilating them, as they are very soft and easily distorted or torn. On the lower surface of the styles near the apical third is a toothed process which is characteristically hidden in some species. If pressure is placed upon the style this tooth may become exposed and may thus destroy the value of that set of genitalia. A drawing of the dorsal aspect of the genitalia is next made as shown in Plate I. The styles should be lying horizontally to give an accurate view. The next step is to draw a lateral view of the connective and œdagus as shown in Plate I. Both outer and inner parts of the genitalia should be preserved in a small vial of alcohol or in a balsam mount.

KEY TO GROUPS.[4]

PAGE

A. M-Cu cross-vein present (Pl. I, fig. 1).
  B. Base of cell M_4 curved (Pl. I, fig. 1); two posteriorly diverging vittæ usually present upon vertex and pronotum. *Obliqua* group.. 109
  BB. Base of cell M_4 angulate (Pl. I, fig. 3); vertex and pronotum not marked as above.
    C. Light-colored median vitta on vertex, pronotum and scutellum; cross-veins whitish; dark-colored species. *Vulnerata group*.. 115
    CC. Vertex, pronotum and scutellum not marked as above; usually light-colored species. *Scutelleris* group.. 118
AA. M-Cu cross-vein absent,[5] media 3 + 4 and cubitus forming a more or less continuous line (Pl. I, fig. 2).
  B. Base of cell M_4 oblique; apex of cell R_3 without black spot. *Maculata* group.. 122
  BB. Base of cell M_4 square; apex of cell R_3 marked with black spot or cloud. *Comes* group.. 129

THE *OBLIQUA* GROUP.

This group is characterized by having the base of cell M_4 made up of the m-cu cross-vein and part of cubitus, the two parts joining together to form a smoothly curving line. (Pl. I, fig. 1.) The color pattern consists of oblique reddish vittæ which may be fused to make a more or less solid area upon the tegmina, or of an inverted V-shaped mark upon the vertex and pronotum. The pygofer hooks are also very characteristic of this group, being short, thick, pointed at the apical end, and bearing a short stublike process beneath.

4. This and some of the following keys are adapted from those in the work of W. L. McAtee, previously referred to.

5. In both of the following groups abnormal cases occasionally occur where there appears to be a short cross-vein present between media and cubitus. This is caused, presumably, by cubitus and media 3 +4 not meeting directly and forming a continuous line as is shown in Plate I, figs. 2 and 4. However, upon comparing the different types of venation shown in the plate referred to and the other characters given for the groups, there should be little difficulty in placing correctly any abnormal individuals.

The group contains some species which are very abundant in individuals but they are not in general known to be especially injurious to economic plants. It includes ten species and four varieties.

KEY TO SPECIES.

A. Principal color markings on tegmina longitudinal.
  B. Longitudinal vittæ usually narrow and paralleling claval suture.
    C. An inverted V-shaped vitta on vertex and pronotum.
      D. Vittæ on clavus and corium similar in color. *obliqua*.. 110
      DD. Vittæ on clavus and corium dissimilar in color. *torra*.. 113
    CC. Vertex, and sometimes pronotum, dusky.
      D. Tegmina dusky-hyaline, vittæ dark red and somewhat obscured. *kanza*.. 112
      DD. Tegmina yellowish, vittæ plainly visible. *fulvocephala*.. 113
  BB. Longitudinal vittæ fused to form a broad dorsal stripe. *dorsalis*.. 112
AA. Principal color markings on tegmina transverse or covering most of surface.
  B. Markings transverse or covering basal half of tegmina, not back to cross-veins.
    C. Narrow red band across middle; pronotum and scutellum red. *rubroscuta*.. 114
    CC. Red to dusky band across basal half and including scutellum. *crevecoeuri*.. 114
  BB. Color covering most of tegmina back to cross-veins.
    C. Tegmina wholly dark red back to cross-veins, except yellow streak on costal margin; vertex and scutellum dark yellow; pronotum mostly brownish-red. *rubricata*.. 114
    CC. Tegmina otherwise marked.
      D. Tegmina reddish with brownish iridescence, except yellow costal plaque and dusky apical cells. *brundusa*.. 112
      DD. Tegmina nearly to cross-veins pinkish-red to dusky-hyaline with pinkish cast, scutellum and anterior parts yellow or dusky. *abolla*.. 113

Erythroneura obliqua Say is a common and distinctive species. It is found in large numbers in the spring, along with several other species, on wild plants growing about woodlands. It, however, does not appear to be injurious to cultivated plants.

Four well-marked varieties occur, and they may be separated by the following key:

KEY TO VARIETIES OF *OBLIQUA*.

A. Tegmina whitish-hyaline to yellow throughout, with oblique red vittæ.
  B. Black spots lacking in basal angles of scutellum.
    C. Oblique red vitta on both clavus and corium. var. *obliqua*.. 111
    CC. Vitta on clavus only and so broadened as to give the clavus an almost entirely reddish color. var. *clavata*.. 112
  BB. A conspicuous black spot in each basal angle of scutellum, sometimes adjoining parts smoky. var. *noevus*.. 111
AA. Tegmina clouded with fulvous.
  B. Tegmina with broad longitudinal dusky vittæ; apical cells M_2 and M_1 abruptly hyaline. var. *electa*.. 111
  BB. Dusky markings in form of cross-bands; apical cells uniformly dusky. var. *fumida*.. 111

Erythroneura obliqua Say.

Tettigonia obliqua Say. Jour. Acad. Nat. Sci., Phila., IV, p. 342; 1825. Reprint, Complete Writings, II, p. 259; 1859.

Color Pattern. Ground color of vertex, pronotum and scutellum yellow, of tegmina whitish, with the following reddish markings: On vertex and pronotum two posteriorly diverging vittæ, forming an inverted V-shaped mark. Scutellum with a median pale yellow vitta bordered by two reddish lines, tip orange-red. Tegmina with an oblique vitta on clavus and another on corium close to claval suture and a streak along costal margin. Veins irregularly marked with red.

Inner Male Genitalia. (Pl. II.)

Host Plants. Plum, both wild and cultivated; also grape and apple. Plums seem to be the favorite.

Distribution. A common and widely distributed species, being reported from Ontario, Maine, Connecticut, Maryland, South Carolina, Virginia, Ohio, Tennessee, Louisiana, Michigan, Illinois, Iowa, Missouri, Nebraska, Kansas, Colorado, California.

Erythroneura obliqua var. *noevus* Gillette.

Typhlocyba obliqua var. *noevus* Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 757; 1898.

Color Pattern. Similar to typical *obliqua*, except that the scutellum has a black spot in each basal angle and sometimes the tip of scutellum and the posterior margin of pronotum are blackish.

Distribution. Ontario, Connecticut, Maryland, Indiana, Illinois, Tennessee, Kansas, Nebraska, Colorado, California.

Erythroneura obliqua var. *fumida* Gillette.

Typhlocyba obliqua var. *fumida* Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 758; 1898.

Color Pattern. Similar to typical *obliqua*, except that it has three broad, dark, transverse bands, one involving the posterior part of the pronotum and anterior part of scutellum; the second across middle of tegmina; the third band very dusky over the cross-veins.

Distribution. Connecticut, Maryland, Virginia, Indiana, Illinois, Kansas, California.

Erythroneura obliqua var. *electa* McAtee.

Erythroneura obliqua var. *electa* McAtee. Trans. Am. Ent. Soc., XLVI, p. 282; 1920.

Color Pattern. "The vittæ on head and pronotum of this variety lack the usual high colors and vary from dull greenish-yellow to slaty; the tegminal vittæ are about typical in form, but are duller red than in variety *obliqua*, and are somewhat obscured by the general duskiness of tegmina, to which, however, the following areas are exceptions: Whitish costal plaques (when developed), two long, triangular areas on inner margin of clavus; and separated very sharply, the clear third and fourth apical cells. General color of body slaty margins of abdominal segments pale greenish-yellow." (McAtee.)

Distribution. Virginia, Illinois, Kansas.

Erythroneura obliqua var. *clavata* DeLong.

Typhlocyba obliqua var. *clavata* DeLong. Bul. No. 17, vol. V, No. 2, Tenn. State Bd. Ent.; 1916.

Color Pattern. "Varying from typical *obliqua* only in coloration. Vertex and pronotum red, anterior margin of each, median line on vertex, and median anterior spot on pronotum yellow. Scutellum and entire claval area bright red, the remainder of elytra uniform yellow to apical area, which is milky white. The red and yellow areas are separated in a sharp line on the claval suture.

"A single specimen was taken at Clarksville, June 29, but is so distinctly marked that it is thought best to class it as a variety." (De Long.)

Distribution. Tennessee.

Erythroneura dorsalis Gillette.

Typhlocyba obliqua var. *dorsalis* Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 757; 1898.
Erythroneura dorsalis Lawson. Sc. Bul. Univ. of Kans., vol. XII, No. 1, 1920.

Color Pattern. White to yellow, with a broad, median, red or black stripe extending from the tip of vertex to, or nearly to, the dusky apical cells.

Inner Male Genitalia. The œdagus differs from all other species in this group in having two pairs of bristlelike structures arising under the base. (Pl. II.)

Distribution. Ontario, Connecticut, Maryland, Virginia, North Carolina, Tennessee, Missouri, Illinois, Minnesota, Nebraska, Kansas, California.

Erythroneura brundusa Robinson.

Erythroneura brundusa Robinson. Can. Ent., vol. LVI, No. 7, p. 155; 1924.

Color Pattern. Vertex red, with anterior margin yellow. Pronotum, lateral and anterior margins yellow; disk smoky, bounded on each side by an oblique maroon vitta. Scutellum brownish-red, with a short white median vitta. Tegmina dark maroon, with bronze iridescence grading down to semihyaline in region of cross-veins. Costal plaque opaque-white. Apical cells dark-smoky.

Distribution. Kansas.

Erythroneura kanza Robinson.

Erythroneura kanza Robinson. Can. Ent., vol. LVI, No. 3, p. 58; 1924.

Color Pattern. Vertex and pronotum smoky-grey to fulvous, with two light red vittæ making an inverted "V." Scutellum red with a brown spot in each basal angle and a white median vitta. Tegmina smoky-hyaline throughout, almost dusky, two dull maroon vittæ each paralleling the claval suture, one on the clavus and the other on the corium; apical cells smoky; cross-veins white.

Inner Male Genitalia. The apical tooth on each style is long and incurved. It differs markedly from all other species except *fulvocephala*. In the latter case, however, the tooth is very much longer, and the apical third of the style is noticeably extended laterally. (Pl. II.)

Distribution. Kansas, Nebraska.

This species is commonly confused with *obliqua* var. *fumida*. However, it bears no striking resemblances to the latter, which has

smoky cross-bands upon a conspicuous white background, while *kanza* is dusky-hyaline throughout with deep maroon vittæ. In the spring it is exceedingly abundant in Kansas upon wild gooseberry, and it is probably of widespread occurrence.

Erythroneura fulvocephala Robinson.

Erythroneura fulvocephala Robinson. Can. Ent., vol. LVI, No. 7, p. 155; 1924.

Color Pattern. Vertex smoky-yellow to fulvous, eyes black. Pronotum smoky-grey with two orange vittæ on disk. Scutellum smoky-yellow. Tegmina whitish-hyaline, with a red vitta on each side, and running nearly full length of claval suture.

Inner Male Genitalia. The apical tooth of the styles is much longer and the apical third of the styles is more extended than in *kanza*. The connective, œdagus, and pygofer hooks also show distinct dissimilarities. The genitalia of *fulvocephala* are much larger than those of *kanza*. (Pl. II.)

Distribution. Kansas.

This species somewhat resembles *obliqua*, but may be separated chiefly by the vertex and pronotum being smoky-yellow to fulvous, while *obliqua* has a reddish inverted V-shaped mark.

Erythroneura torra Robinson.

Erythroneura torra Robinson. Can. Ent., vol. LVI, No. 7, pp. 155; 1924.

Color Pattern. Vertex and pronotum yellow, with a red median inverted V-shaped vitta. Scutellum, a brown spot in each basal angle; a white median vitta outlined in red. Tegmina hyaline, a broad red vitta on clavus and a pale orange vitta on corium, both paralleling claval suture; costal margin yellowish.

Inner Male Genitalia. The apical projection of the œdagus is long, narrow and straight, while in *obliqua*, which this species somewhat resembles in color pattern, the process is short and curved. The styles of the two species also show marked differences. (Pl. II.)

Distribution. Kansas.

This species has some resemblances to *obliqua*, but may be separated easily by the fact that *torra* has a broad reddish vitta on the clavus and a narrow pale yellow one on the corium, while with *obliqua* the two vittæ are of the same color.

Erythroneura abolla McAtee.

Erythroneura abolla McAtee. Trans. Am. Ent. Soc., XLVI, p. 285; 1920.

Color Pattern. "The general color varies from diffuse pinkish-red to dusky-hyaline with a pinkish cast, the color extending to, or nearly to, cross-veins, beyond which the tegmina are hyaline more or less clouded with whitish to dusky; costal plaques whitish opaque. The scutellum and anterior parts in some cases are yellowish, and the red color on tegmina may be almost entirely restricted to the veins, along which it tends to form streaks. Face varying

from yellowish to pinkish red, sometimes with median vitta and underside of vertex paler; legs pale, they and pleura more or less tinged with same color as face; body slaty with margins and incisures of abdominal segments, and genitalia chiefly paler." (W. L. McAtee.)

Inner Male Genitalia. The genitalia in general are similar to *obliqua*. However, the apical tooth of the style and the apical extension of the œdagus show some slight differences. (Pl. II.)

Distribution. Maryland, Virginia, Illinois, Kansas, California.

This species was placed by McAtee in another group because the base of cell M_4 is inclined to be somewhat angulate instead of smoothly curving; however, when the inner male genitalia are studied the fact is at once revealed that *abolla* belongs to this group.

Erythroneura rubricata Van Duzee.

Typhlocyba rubricata Van Duzee. Bul. Buf. Soc. Nat. Sc., IX, p. 229; 1909.

Color Pattern. Vertex and scutellum dark yellow; pronotum sometimes yellowish on anterior margin, remainder brownish-red. Tegmina reddish to brownish-red, a streak along costal margin, involving costal plague, yellow. Apical cells slightly dusky.

Distribution. Florida.

I have not been able to examine the inner genitalia of this species. However, the characters of the wing venation show that it belongs to the *Obliqua* group.

Erythroneura rubrosceuta Gillette.

Typhlocyba rubrosceuta Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 755; 1898.

Color Pattern. Vertex pale yellow, usually with red spot at tip. Pronotum mostly reddish, with a yellow spot on middle of anterior margin. Scutellum red. Tegmina whitish-hyaline, with a red band across the apical third on clavus to, or almost to, costal plaque. A red streak on costal margin near base and on cross-veins.

Inner Male Genitalia. The œdagus is elongate and slender throughout, and the apical process is long and upward curving; the apical third of the style is stout and has a forward projecting tooth. (Pl. II.)

Distribution. Ohio, Illinois, Kansas.

Erythroneura crevecoeuri Gillette.

Typhlocyba crevecoeuri Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 767; 1898.

Color Pattern. Vertex and pronotum pale yellow, with a reddish inverted V-shaped mark. Scutellum dark red. Tegmina, basal two-thirds deep red with middle portion often dusky, apical third pale yellow. Cross-veins and adjacent parts of longitudinal veins sometimes red.

Inner Male Genitalia. The apical third of the style is attenuate; the œdagus is slender and trifurcate at the apical end. (Pl. II.)

Distribution. New York, Connecticut, North Carolina, Ohio, Kansas.

THE *VULNERATA* GROUP.

In this group the base of cell M4 is angulate; all the species have a light-colored median vitta upon the vertex, pronotum and scutellum; the cross-veins, and frequently the adjacent parts of longitudinal veins, are whitish; and the species are all dark colored. There are four species and three varieties in this group.

KEY TO SPECIES.

		PAGE
A.	Color of upper surface ranging from greenish-brown to brownish-maroon, not blackish.	
B.	Markings in basal half of tegmina orange, in apical half light yellow; median vitta inconspicuous, light spots on margin of pronotum.	*gradata*.. 117
BB.	Markings otherwise.	
C.	Dorsum deep brown with small conspicuous grey spots; large black area in each basal angle of scutellum.	*pulchella*.. 117
CC.	Dorsum greenish-brown, brown or maroon.	*vulnerata*.. 115
AA.	Dorsum very dark to black throughout.	*niger*.. 116

Erythroneura vulnerata Fitch, while found commonly on the grape, is seldom abundant enough to be considered a serious pest. The nymphs in the early stages are conspicuously colored with bright orange upon a creamy ground color, and in the later stages they become deep red. The nymphs feed with the tip of the abdomen turned upward. The adults are often found on the upper surface of the grape foliage, and this distinguishes them generally from the other species. Their darker color harmonizes with that of the foliage and renders them less conspicuous.

KEY TO VARIETIES OF *VULNERATA*.

		PAGE
A.	Tegminal color markings chiefly smoky-brown to black, pale areas conspicuous by contrast.	var. *decora*.. 116
AA.	Tegminal color markings otherwise, pale areas less conspicuous.	
B.	Markings of scutellum and anterior parts about the same color as those of tegmina.	*vulnerata*.. 115
BB.	Markings of scutellum and anterior parts smoky to black, of tegmina vivid maroon.	var. *fulmina*.. 116

Erythroneura vulnerata Fitch.

Erythroneura vulnerata Fitch. N. Y. St. Cab. Nat. Hist., pp. 62, 63; 1851.

Color Pattern: Ground color greenish-brown to fulvous or maroon, relieved by the following pale areas: a median vitta more or less continuous over vertex, pronotum and scutellum; a vitta bordering each eye. Pronotum with two or more spots on each side. Tegmina with whitish spots on clavus and corium; costal plaque yellow with an oblique black mark at each end. Cross-veins and adjacent parts of longitudinal veins whitish. Apical cells smoky.

Inner Male Genitalia. The pygofer hooks are weakly bifurcate at the apex and have a prominent ventral stub; dorsally, the œdagus bears three prominent teeth, one straight and two strongly curved. (Pl. III.)

Host Plants. Grape, cultivated and wild; *Clematis* spp., *Ampelopsis* spp.

Distribution. Occurs in Ontario and Quebec, Canada; and widely distributed throughout the United States.

Erythroneura vulnerata var. *fulmina* McAtee.

Erythroneura vulnerata var. *fulmina* McAtee. Trans. Am. Ent. Soc., XLVI, p. 274; 1920.

Color Pattern. "Scutellum and anterior parts with color markings chiefly smoky to black, pale areas much reduced; tegmina with ground color opaque whitish, color markings pinkish to maroon, ground color of apical cells black. A most beautiful variety, the dark fore and hind parts contrasted with the paler mid-section, the latter in turn colored with beautifully contrasting milky white and dark red, the costa flushed and the sectors and first cross-vein dotted with bright pinkish red." (W. L. McAtee.)

Distribution. Maryland, Kansas.

Erythroneura vulnerata var. *decora* McAtee.

Erythroneura vulnerata var. *decora* McAtee. Trans. Am. Ent. Soc., XLVI, p. 274; 1920.

Color Pattern. "Color markings smoky to black, pale areas conspicuous by contrast, and much larger than in variety *vulnerata*, vertex pale yellow with two broad black vittæ inclosing a narrow median pale one, pronotum with median and two discal pale yellow spots, sometimes merged, spots and dashes of same color near lateral and anterior margins; median scutellar vitta broad, and basal triangles paler within; tegminal pale areas large, that on base of clavus conspicuous, pale yellow; costal plaque pale yellow." (McAtee.)

Distribution. Maryland.

Erythroneura niger Gillette, placed as a variety of *vulnerata*, has been found by the study of its inner male genitalia to be a distinct species; *vulnerata* var. *nigerrima* McAtee belongs to this species.

KEY TO VARIETIES OF *NIGER*.

		PAGE
A.	Clavus with a single whitish yellow basal spot.	*niger*.. 116
AA.	Clavus without a large yellow basal spot.	var. *nigerrima*.. 117

Erythroneura niger Gillette.

Typhlocyba vulnerata var. *niger* Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 765; 1898.

Typhlocyba nigridorsum DeLong. Bul. 17, vol. V, No. 2, Tenn. State Bd. Ent.; June, 1916.

Erythroneura nigra Lawson. Sc. Bul. Univ. of Kans., vol. XII, No. 1; 1920.

Color Pattern. Color dark brown or black, with the following white markings: Vertex, pronotum and scutellum each with a pale median vitta, the first two sometimes reduced to discal spots. Tegmina, a large spot on inner margin at base of clavus; other pale areas much reduced. Cross-veins broad and pale.

Inner Male Genitalia. The pygofer hooks consist of a sharp process of medium length with a very broad and heavy base, which is unlike the hooks of the other species. The œdagus shows marked dissimilarities and the genitalia in general differ considerably from those of the other species of this group. However, *niger* shows a relationship in the matter of color pattern and wing venation. (Pl. III.)

Distribution. New York, Connecticut, Maryland, Virginia, Tennessee, Illinois, Iowa, Nebraska, Kansas, Colorado.

This species and its variety can be distinguished by their dark, almost black, coloration. It probably breeds upon the grape.

Erythroneura niger var. *nigerrima* McAtee.

Erythroneura vulnerata var. *nigerrima* McAtee. Trans. Am. Ent. Soc., XLVI, p. 275; 1920.

Color Pattern. "A darker form even than variety *niger*, the pale areas on clavus being smaller, semi-hyaline and inconspicuous; in extreme examples there is only a single pale point at two-thirds length of clavus, and one on corium near it, besides the pale yellow costal plaque and paler costal area just posterior." (W. L. McAtee.)

Distribution. Maryland, Virginia, Illinois, Iowa, Kansas, Colorado.

Erythroneura gradata Robinson.

Erythroneura gradata Robinson. Can. Ent., vol. LVI, No. 3, p. 58; 1924.

Color Pattern. Vertex and pronotum yellow, with sometimes an elongate red vitta on each side of a pale median line. Scutellum yellow with paler median vitta. Tegmina, ground color white-hyaline, basal half of clavus red except a mark resembling an inverted comma at inner basal angle; an irregular orange-red area on corium extending from humerus half the length of claval suture; apical third of clavus and the veins up to the whitish cross-veins light orange yellow; a smoky area extending from the tip of cell R_3 to the base of cells M_2 and M_4; costal plaque yellow to white with posterior oblique dark line.

Inner Male Genitalia. The apex of the pygofer hooks is strongly and widely bifurcate; the ventral stub is absent; and the base is broad, flat and obliquely inclined. (Pl. III.)

Distribution. Kansas, Colorado.

In *gradata*, the anterior median light vitta is less conspicuous than in the other species, and frequently there is a reddish vitta on each side. The red coloration of the basal part of the tegmina becomes orange to yellow beyond the clavus. However, this species is easily separated on the above characters from *vulnerata* which it most nearly resembles.

Erythroneura pulchella Robinson.

Erythroneura pulchella Robinson. Can. Ent., vol. LVI, No. 7, p. 155; 1924.

Color Pattern. Vertex light brown, with anterior margin, a median vitta enlarged at each end, and a lateral spot opposite each eye, yellowish; all these marks outlined in dark brown. Pronotum light brown, with a blackish ring on disk and two grey spots on posterior margin; five gray spots outlined in dark brown arranged around lateral and anterior margins. Scutellum light brown, with a black triangular spot in each basal angle and a median white vitta outlined in brownish-red. Tegmina, clavus chocolate-maroon except two spots near base and one in apical third greyish; corium grey, with dashes of brown between the longitudinal veins, the latter of which are whitish dotted with

red; costal plaque whitish on inner and red on outer margins and having a black streak at each end; cross-veins and apical veins pinkish-white; apical cells smoky-brown.

Distribution. Kansas.

THE *SCUTELLERIS* GROUP.

In this group there is a wide range of color pattern, but all agree in not having a pale median vitta upon the vertex, pronotum and scutellum, and all are much lighter colored species than in the previous group. The cross-veins are also not conspicuously whitish. It is a rather heterogeneous group, and contains all those species having the base of cell M_4 angulate and not included in the *vulnerata* group.

KEY TO SPECIES.

A. Apical cell M_4 bearing a black cloud or dot at base.
  B. Tegmina brilliant red with a large white discal area. *hartii*.. 119
  BB. Tegmina colored otherwise.
    C. Vertex and scutellum conspicuously spotted with red or black.
      D. Spots red, single; chief color markings of tegmina black dots. *illinoiensis*.. 119
      DD. Spots black, double; tegmina brownish with whitish areas. *tecta*.. 121
    CC. Vertex and scutellum not as above.
      D. Principal markings two conspicuous black bands, one across pronotum and the other at base of apical cells. *morgani*.. 119
      DD. Principal markings otherwise.
        E. Scutellum and most of pronotum smoky-brown or black, remainder whitish-yellow with red or yellowish markings. *scutelleris*.. 118
        EE. Scutellum and pronotum not smoky-brown or black.
          F. Color pattern rose (sometimes dusky) throughout and covering most of tegmina. *rosa*.. 120
          FF. Color pattern bright red on anterior half and orange on posterior and occurring as more or less isolated areas. *rubraza*.. 121
AA. Apical cell M_4 without a black cloud or dot at base.
  B. Entire upper surface pale yellow. *ador*.. 120
  BB. Upper surface otherwise.
    C. Vertex and scutellum each with two distinct black spots. *bipunctata*.. 121
    CC. Color pale yellow with orange-yellow vittæ; female genital plate produced and notched. *dentata*.. 120

Erythroneura scutelleris Gillette.

Typhlocyba comes var. *scutelleris* Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 764; 1898.

Color Pattern. Vertex varies from reddish with five yellow spots to dusky-yellow. Pronotum varies from yellow with reddish markings anteriorly and smoky-brown posteriorly to deep brown throughout, with a small yellow dot on middle of anterior margin. Scutellum smoky-brown to black. Tegmina, whitish-hyaline or yellow, with the following red markings: Three spots on clavus, the first two sometimes joined to form a barbed vitta, spots on corium irregularly arranged around the costal plaque. Cross-veins and adjacent parts of longitudinal veins reddish.

Inner Male Genitalia. The pygofer hooks are long and slender and have a stout tooth at the apical end. (Pl. IV.)

Distribution. Connecticut, Maryland, Virginia, Tennessee, Ohio, Missouri, Kansas.

Erythroneura morgani DeLong.

Typhlocyba morgani DeLong. Bul. 17, vol. V, No. 2, p. 104, Tenn. State Board of Entomology; 1916.

Color Pattern. "Pale yellowish, eyes pale fuscous, pronotum with a rather broad brownish-black band across posterior two-thirds; disk, anterior margin before it, and a point behind either eye whitish. Elytra pale, marked with slightly darker yellow spots, and a broad band before nervures of apical cells, brown. Beneath pale." (D. M. DeLong.)

Inner Male Genitalia. The pygofer hooks are elongate, slightly curved and sharply toothed apically; and there are two widely separated fingerlike processes at the base. The styles are very slender apically with a long drawn-out tooth which bends midway to form a right angle. (Pl. III.)

Distribution. Maryland, Ohio, Tennessee, Illinois, Kansas.

Erythroneura hartii Gillette.

Typhlocyba hartii Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 754; 1898.

Color Pattern. Vertex, pronotum and scutellum pale yellow to white, sometimes with faint orange marks. Tegmina brilliant red to cross-veins except a large discal spot of ivory-white, and dashes of white on costal margin. Apical cells yellowish-white.

Inner Male Genitalia. The pygofer hooks are spearlike with two closely approximated fingerlike processes at the base. The œdagus bears a dorsal projection with three sharply pointed teeth. (Pl. III.)

Host Plants. It is reported as being a pest of young apple trees.

Distribution. Connecticut, Pennsylvania, Virginia, Indiana, Illinois.

Erythroneura illinoiensis Gillette.

Typhlocyba illinoiensis Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 758; 1898.

Color Pattern. Vertex, pronotum and scutellum white to yellowish-white and each bearing a large red spot. Tegmina whitish-hyaline to yellow, with a large black spot on the corium nearly midway along the claval suture. Frequently this spot outlined in red, and with red dots at base of the corium, base and tip of clavus, and streaks along the longitudinal veins. A black dot at the apex of the costal plaque and in the base of cell M_4.

Inner Male Genitalia. (Pl. III.)

Host Plants. Grapes, both wild and cultivated.

Distribution. New York, Connecticut, Maryland, Virginia, North Carolina, Tennessee, Indiana, Michigan, Illinois, Mississippi, Kansas.

Erythroneura rosa Robinson.

Erythroneura rosa Robinson. Can. Ent., vol. LVI, No. 3, p. 58; 1924.

Color Pattern. Vertex dull red, with two yellow spots on anterior margin and three on posterior. Pronotum slaty to maroon, three pallid spots on anterior margin, the two outer ones being longer than the inner; discal area slaty. Scutellum light yellow with basal angles brown and tip red. Tegmina whitish-hyaline with the following color pattern: Whole of clavus pale rose, except a white area at the inner basal angle adjoining tip of scutellum and one in posterior third but not involving the tip; in the middle of clavus the color sometimes darker red. This coloration extends across corium to the yellow costal plaque in a broad band about as wide as long from which a narrow oblique streak extends back to cells M_4 and M_2. Base of apical cells smoky; a dark spot in apex of costal plaque.

Inner Male Genitalia. The pygofer hooks extend downward from their point of attachment, bend to form a right angle and end in a long slender spine. The styles are attenuate apically and have a short tooth which is not readily seen. (Pl. III.)

Host Plants. This species appears to be common on nettles (*Urtica gracilis*), which may be found to be its host plant.

Distribution. Missouri, Kansas.

Erythroneura ador McAtee.

Erythroneura ador McAtee. Notes on Nova Scotian Eupterygid Leaf-hoppers, including descriptions of two new species. Can. Ent. XXIX, No. 9, p. 361; Nov., 1918. (Halifax, Nova Scotia.)

Color Pattern. "An unusually large species for the genus, with long but blunt vertex and long and wide fourth apical cell, angulate at base. Color pale yellow, tegmina varying through greenish to golden-yellow, paler apically; costal plaques somewhat denser yellow; eyes brownish-yellow; clavus dark." (McAtee.)

Distribution. Nova Scotia.

I have not been able to examine the genitalia of this species.

Erythroneura dentata Gillette.

Typhlocyba dentata Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 765; 1898.

Color Pattern. ". . . (vertex) pale yellow and having two illy defined orange or lemon-colored spots which, in one specimen, extend forward over the crest of the vertex. Pronotum pale yellow, with two rather large and approximate orange-colored spots just before the middle. Scutellum light yellow on the middle, but more or less washed with orange on all the angles. Elytra light yellow, subhyaline on the basal portion, transparent beyond the cross veins and with two orange vittæ, one on the clavus and another longitudinal one on the middle of the corium and reaching to the cross veins. . . . The last ventral segment of the female has a large broad tooth notched at the tip. . . . The orange coloration is quite faint in two of the specimens." (Gillette.)

Distribution. Illinois, California.

I have not been able to examine the genitalia of this species.

Erythroneura bipunctata Gillette.

Typhlocyba bipunctata Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 751; 1898.

Color Pattern. "Vertex red, with two conspicuous black spots a little before the middle surrounded by a narrow band of pale yellow; length about one-third greater at the middle than at the eyes. Pronotum red, with four small pale spots on the anterior margin, posterior margin straight. Scutellum red, with a black spot on each basal angle and with pale coloration margining these spots and upon the apex. Elytra red with a line on claval suture and one on the corium beneath white, beyond cross nervures smoky subhyaline, nervures red." (Gillette.)

Inner Male Genitalia. The apex of the styles is unusually short, and the part which precedes it is especially broad. The connective and the œdagus are very heavy. The pygofer hooks arise from a heavy base and turn abruptly upward. (Pl. IV.)

Distribution. Texas, Arizona.

Individuals of this species occur with the markings smoky-brown or greenish-yellow instead of red.

Erythroneura rubraza Robinson.

Erythroneura rubraza Robinson. Can. Ent., vol. LVI, No. 12, p. 291; 1924.

Color Pattern. Vertex ivory on anterior margin, remainder red and inclosing three ivory spots. Pronotum, ivory on anterior margin, remainder white; a red discal area with two anterior projections of yellow, and a small lateral spot of red behind each eye. Scutellum yellow, with a red spot in each angle. Tegmina whitish-hyaline and marked as follows: A red spot at base of clavus, another at tip and a narrow band of same color across middle; a red spot at base of corium, and a red line beginning in front of costal plaque proceeding obliquely inward, where by a short extension it touches middle of claval suture; the line then becomes faint orange and proceeds backward between media and cubitus the length of the costal plaque, where it increases greatly in width and covers remainder of tegmina to cross veins with a faint wash of orange, which is especially noticeable on the longitudinal veins. The costal margin and radius are bright red. A small black spot posterior to costal plaque and a large one in base of cell M_4.

Inner Male Genitalia. The pygofer hooks are very long and slender, with a shorter hook arising ventrally from the basal third. The connective is narrow at the base and broadens greatly toward the apex of each arm. (Pl. III.)

Distribution. Kansas.

Erythroneura tecta McAtee is "a very distinctly marked species, larger than most of the genus, with bluntly rounded vertex, swollen face, pronotum decidedly arcuate posteriorly, clavus distinctly roof-shaped, the part within claval (second anal) vein lying horizontally, when tegmina are closed, and that without sloping sharply; fourth apical cell angulate; ventral plate of female arcuate laterally, much produced medianly, this process more or less split longitudinally." (McAtee.)

KEY TO VARIETIES OF *TECTA*. PAGE

A. Averaging larger (2.9 to 3.46 mm.); pronotum and scutellum chiefly yellow; tegmina usually with considerable red markings. var. *tecta*.. 122

AA. Averaging smaller (2.64 to 2.97 mm.); pronotum and scutellum chiefly dusky; tegmina with few or no red markings. var. *carbonata*.. 122

Erythroneura tecta var. *tecta* McAtee.

Erythroneura tecta var. *tecta* McAtee. Trans. Am. Ent. Soc., XLVI, p. 289; 1920.
Erythroneura serpunctata Malloch. Bul. Brook. Ent. Soc., 16, p. 25; 1920.

Color Pattern. "Ground color of scutellum and anterior parts pale yellow; vertex with two round dusky spots on disc which are more or less connected with each other, with inner side of orbits, and with posterior margin by arcuate reddish markings; pronotum with an angulate brown vitta, sometimes broken up into dusky spots on each side, and two discal spots sometimes concealed by reddish markings, which when fully developed form a "U" based on posterior margin; scutellum with basal triangles black, margins and apex reddish; tegmina whitish-hyaline (dorsal pale areas more or less opaque); clavus is perhaps best described by saying it is red, sometimes brownish, except the whitish-hyaline extreme base, a large more opaque whitish or pale yellow area near scutellum and entirely within claval (second anal) vein, a smaller whitish area overlapping apex of last but entirely outside claval vein, and a small transverse whitish band just before apex; corium more or less bluish or dusky between veins, the veins often reddish; two whitish areas bounded by red between third sector and claval suture, costal plaque pale yellow, more or less overlaid by opaque white, bounded at both ends by dusky clouds, costa hyaline posteriorly, interrupted by red cross-vein; apical cells fumose, with a darker area at base of fourth cell and another at apex of wing, common to second and third cells, often forming an oblique vitta. Face pale yellowish, a dark spot on base of clypeus, two others above just within antennal bases, lower surface of vertex marked by a few reddish lines; legs pinkish livid, body slaty, or with pale yellowish edgings." (W. L. McAtee.)

Inner Male Genitalia. The pygofer hooks consist of a broad, heavy base and a slender apically projecting spine much like those of *niger*. (Pl. IV.)

Distribution. Maryland, Illinois.

Erythroneura tecta var. *carbonata* McAtee.

Erythroneura tecta var. *carbonata* McAtee. Trans. Am. Ent. Soc., XLVI, p. 289; 1920.

Color Pattern. "A smaller, dusky form, the scutellum and pronotum especially being much darker than in the typical variety, the tegmina with much less red, the tip of clavus and margin of pale spot near base of clavus, however, sometimes red." (McAtee.)

Distribution. Maryland.

THE *MACULATA* GROUP.

In this group cubitus turns abruptly forward in the region of the "cross-veins," and thus forms an oblique base for cell M_4. This cell always bears a black spot in the base. In cell R_3 a black spot is lacking, and this serves partly to separate this group from the *comes* group. The pygofer hooks are long and spear-like.

KEY TO SPECIES OF *MACULATA* GROUP.

A. Color pattern made up chiefly of yellow to red spots; black dot in base of cell M_4 large and conspicuous.
  B. Ground color of dorsum deep ivory; spots very dark red. *maculata*.. 123
  BB. Ground color of tegmina light yellow to hyaline; spots light red to yellow.
    C. Tegmina hyaline; spots small, very faint red and lacking a definite outline. *hymac*.. 126
    CC. Tegmina yellowish-white; spots larger and with definite margins.
      D. Spots light orange to yellow; area in middle of clavus tends to be rectangular; color pattern confined chiefly to tegmina. *lawsoni*.. 125
      DD. Spots bright red; mid-claval area concave on anterior and convex on posterior margins; scutellum and anterior parts marked with red. *mediana*.. 126
AA. Color pattern made up chiefly of red zigzag vittæ or of conspicuous red to smoky bands across clavus and corium; black dot in base of cell M_4 usually small and inconspicuous.
  B. Pattern chiefly transverse band or bands.
    C. Cross bands three in number, black, involving base, middle and apex of tegmina, respectively. *trivittata*.. 127
    CC. Cross band one in number.
      D. Band very broad, chiefly smoky-brown and covering scutellum and basal half of tegmina, sometimes involving most of pronotum. *basilaris*.. 127
      DD. Band narrower and red; not involving scutellum and pronotum.
        E. Band brilliant red across basal third of tegmina; other coloration pale yellow. *osborni*.. 126
        EE. Band very faint red and crossing tegmina near middle of clavus; other coloration consists of pink marks upon a white ground color. *univittata*.. 127
  BB. Pattern chiefly zigzag vittæ.
    C. Vittæ scarlet; of nearly uniform width; continuous from scutellum to cross-veins; tip of clavus lacking a red spot; tegmina semi-hyaline; other color markings much lighter than main vitta. *ligata*.. 128
    CC. Vitta light red; not of uniform width; obviously made up of several vittæ more or less joined together; red spot near tip of clavus; tegmina opaque-white; other color markings of same color as main vitta.
      D. Vittæ very broad, especially from middle of clavus to costal plaque; practically one continuous line from scutellum to cross-veins. *torella*.. 125
      DD. Vittæ narrower; usually disconnected at claval suture and before cross-veins into three vittæ, one on basal half of clavus, one on disk of corium and one before cross-veins. *campora*.. 124

Erythroneura maculata Gillette is one of the species which breeds on the grape. It is a fairly common form, but not so numerous as some of the other grape leaf hoppers, and is not mentioned as being an injurious species.

In this and the following keys I have not included the colorless varieties described by McAtee. It is probable they were newly matured individuals and were captured and killed before their color appeared, which requires a period of several hours after the final molt.

KEY TO VARIETIES OF *MACULATA*.

PAGE

A. Spots on clavus and corium uniformly dark red and of nearly equal size, no part standing out conspicuously. *maculata*.. 124

AA. Clavus with conspicuous red patch or band sometimes extending over the adjacent parts of corium.

B. Anterior two-thirds of clavus red. var. *bella*.. 124

BB. Middle of clavus with a red band which sometimes passes more or less continuously across corium. var. *era*.. 124

Erythroneura maculata Gillette.

Typhlocyba comes var. *maculata* Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 764; 1898.

Color Pattern. General ground color ivory to white, with the following markings in red: Vertex, an irregularly shaped vitta whose arrangement tends to inclose from one to three ivory spots. Pronotum, two lateral and one discal spots. Scutellum, a triangular outline in each basal angle, a spot at tip. Tegmina, on clavus a spot at base, middle and tip, the first two sometimes joined together; on corium, a spot at base, before costal plaque, between plaque and claval suture, and at apex of claval suture. Streaks along cross-veins and adjacent parts of longitudinal veins; apical cells hyaline; a black spot in base of cell M_4.

Inner Male Genitalia. The pygofer hooks in this species consist of two long projections, the lower one slightly the longer of the two. There is a long blunt tooth at the apex of the styles. (Pl. V.)

Host Plants. Grapes, wild and cultivated.

Distribution. Ontario, Connecticut, Maryland, Virginia, Georgia, Illinois, Wisconsin, Nebraska, Kansas, Texas.

Erythroneura maculata var. *bella* McAtee.

Erythroneura maculata var. *bella* McAtee. Trans. Am. Ent. Soc., XLVI, p. 300; 1920.

Color Pattern. "Like the red form of var. *maculata*, except that the anterior two-thirds of the clavus and sometimes a spot on adjoining corium is red." (McAtee.)

Distribution. Maryland.

Erythroneura maculata var. *era* McAtee.

Erythroneura maculata var. *era* McAtee. Trans. Am. Ent. Soc., XLVI, p. 299; 1920.

Color Pattern. "Middle of clavus has a distinct red band, which is extended more or less continuously across corium to front of costal plaque; subsidiary tegminal markings red." (McAtee.)

Distribution. Virginia, Illinois.

Erythroneura campora Robinson.

Erythroneura campora Robinson. Can. Ent., vol. LVI, No. 3, p. 59; 1924.

Color Pattern. Vertex red, with five white spots which tend to be arranged as follows: two on anterior margin, two on posterior, and one central. Ground color of pronotum, scutellum and tegmina creamy white, with the following red marks: On pronotum a Y-shaped median vitta with heavy base, a spot on each side; scutellum, three triangular spots, one in each angle; tegmina, in

basal half of clavus an oblique vitta which is enlarged caudally to face the tegminal suture, near tip a spot, a spot on corium near humeral angle, a more or less continuous semi-circular vitta bordering the inner margins of the costal plaque and extending to the base of cell M_4. Cross-veins and adjacent parts of longitudinal veins red. A black spot in apex of costal plaque and another in base of cell M_1.

Inner Male Genitalia. The pygofer hooks consist of a single long spear-like process. (Pl. V).

Distribution. Ontario, New York, Arkansas, Kansas, Nebraska.

This species is frequently confused with *maculata;* however, the inner male genitalia prove it to be a distinct species. It is easily distinguished by a white instead of ivory ground color, and the markings being light instead of dark red, and elongate instead of broken up into spots.

Erythroneura torella Robinson.

Erythroneura torella Robinson. Can. Ent., vol. LVI, No. 7, p. 156; 1924.

Color Pattern. Ground color of vertex, pronotum and scutellum yellowish, with the following marks in crimson: Vertex, an oval outline on disk with an extension on each side to meet the eye. Pronotum, a median Y-shaped vitta whose arms enclose a yellow circular spot, a lateral streak behind each eye. Scutellum, a triangle in each basal angle and a spot at tip. Tegmina whitish, with the following marks in orange-red: A broad vitta with caudal barb occupying basal half of clavus, and a spot at tip; a spot in base of corium; a mark arising at base of costal plaque, continuing around the inner margin to the apex where it extends inward to the base of cell M_4, then curves outward again to the crimson radius. A small dot in base of cell M_4.

Inner Male Genitalia. The pygofer hooks have a terminal tooth which is characteristically downward turned. The connective is much swollen at the apex of each arm, the remainder unusually slender. The apex of the styles is attenuate and bears a long tooth. (Pl. IV.)

Distribution. Kansas.

Erythroneura lawsoni Robinson.

Erythroneura lawsoni Robinson. Can. Ent., vol. LVI, No. 3, p. 59; 1924.

Color Pattern. Vertex, pronotum and scutellum pale yellow, with the following white markings: Two spots on posterior margin of vertex close to the eyes, a median vitta on disk; a large spot on middle of anterior margin of pronotum and a lighter one on each side, posterior margin whitish; scutellum, a median vitta sometimes not reaching the tip. Tegmina, ground color creamy white with orange markings as follows: On clavus a small spot near base, an almost square area in basal third, and a small spot near tip; on corium an oblique vitta in front of costal plaque, an irregular area between the latter and the clavus; some irregular streaks in front of the red cross-veins. A small black dot in the apex of costal plaque and a conspicuous black spot in base of cell M_4.

Inner Male Genitalia. The apical tooth of the styles is very long and smoothly curving, unlike that of any other species in the group. (Pl. V.)

Host Plants. This species has been taken on sycamore.

Distribution. New York, Arkansas.

Erythroneura mediana Robinson.

Erythroneura mediana Robinson. Can. Ent., vol. LVI, No. 7, p. 156; 1924.

Color Pattern. Vertex, five yellow spots more or less outlined in red and arranged two on anterior and two on posterior margins and one central. Pronotum whitish, with a median Y-shaped vitta and two lateral spots reddish. Scutellum yellow, with an orange spot outlined in red in each basal angle, and tip red. Tegmina opaque-white with the following red marks: Clavus, a spot near base, another near tip and a larger area about the middle, the latter of which having the anterior margin concave and posterior convex. Corium, a spot near base, an oblique streak running from front of costal plaque to near the spot in tip of clavus. Longitudinal veins throughout greater part of their length, and also cross-veins streaked or dotted with red. A black spot in base of cell M_4.

Inner Male Genitalia. The apical tooth of the styles is long and straight except at the base which bears an upward barb. The pygofer hooks are especially long. The dorsal part of the œdagus bears a somewhat triangular projection. (Pl. V.)

Distribution. Kansas.

Erythroneura hymae Robinson.

Erythroneura hymae Robinson. Can. Ent., vol. LVI, No. 3, p. 60; 1924.

Color Pattern. Vertex, two ivory spots on anterior margin and three on posterior, the middle spot faintly encircled with red. Pronotum, ivory, faint red Y-shaped median vitta and a red triangular spot behind each eye. Scutellum, a small red spot in each basal angle and with orange or red tip. Tegmina subhyaline, with eight faint orange spots arranged almost equidistantly as follows: Three on clavus and five on corium; a small black spot behind the region of costal plaque and one in base of cell M_4; cross-veins more or less reddish.

Inner Male Genitalia. The pygofer hooks turn abruptly upward at one-third of their length from the base and continue in a long tapering hook. (Pl. IV.)

Distribution. Kansas.

Erythroneura osborni De Long.

Typhlocyba osborni De Long. Bul. No. 17, vol. V, No. 2, p. 103, Tenn. State Bd. of Ent.; 1916.

Erythroneura maculata var. *osborni* McAtee. Trans. Am. Ent. Soc., XLVI, p. 300; 1920.

Color Pattern. "Very pale yellowish or white, vertex and face unmarked, pronotum with yellowish spot behind each eye, and an orange crescent-shaped spot, posteriorly convex on disc. Scutellum white. Elytra with a broad blood-red band on basal third of elytra, evenly colored, often not reaching costa. A black spot at the middle of costal margin, another on the inner margin be-

fore the cross nervures of the apical cells and a red one before the nervures on the costal margin." (De Long.)

Inner Male Genitalia. The apical half of the pygofer hooks is much enlarged and is terminated by a short tooth. The apex of the styles has a strong lateral projection and bears no visible tooth. (Pl. IV.)

Distribution. Tennessee, Illinois.

Erythroneura trivittata Robinson.

Erythroneura trivittata Robinson. Can. Ent., vol. LVI, No. 3, p. 59; 1924.

Color Pattern. Vertex creamy yellow, with very pale red marks outlining three yellow spots on posterior margin. Pronotum yellow on anterior and greyish on posterior margins, with a small red spot on the latter behind each eye. Scutellum deep blood red. Tegmina, maroon band across base; in the apical third of clavus but not involving the tip a small red band which is continued on to the corium, where it broadens abruptly to about twice the width and the color deepens to a dark maroon; an almost square blue area in the region of the costal plaque carries the band across to the costal margin; cross-veins and parts of adjacent longitudinal veins red; a smoky area extending from the base of cell M_4 to the apex of cells R_3 and R_1.

Inner Male Genitalia. The œdagus bears four projections, which resemble dorsally a Greek cross. At the base of the pygofer hooks are two ventral finger-like processes. (Pl. IV.)

Distribution. Kansas.

Erythroneura univittata Robinson.

Erythroneura univittata Robinson. Can. Ent., vol. LVI, No. 7, p. 156, 1924.

Color Pattern. General ground color of anterior parts yellowish-white, of tegmina whitish-hyaline, with following marks in very pale rose: Vertex, streaks outlining a median vitta and two lateral spots. Pronotum, a median and two smaller lateral spots. Scutellum, a spot at tip. A band arising in front of costal plaque and extending across tegmen, widening as it proceeds inward. A spot at tip of clavus and a streak along cubitus. A black spot behind costal plaque and another in base of cell M_4.

Inner Male Genitalia. The pygofer hooks are exceptionally long and curve downward soon after leaving the heavy base and forward shortly before the terminal tooth. (Pl. IV.)

Distribution. Kansas.

Erythroneura basilaris Say is a well-marked species, especially the dark or typical form. Two varieties occur which lack the dusky area over the base of the tegmina.

KEY TO VARIETIES OF *BASILARIS*.

		PAGE
A.	Color markings, in part, red to sanguineous.	
B.	Basal tegminal band sanguineous to dusky.	var. *basilaris*.. 128
BB.	Basal tegminal bank pinkish, other markings chiefly yellow.	var. *dulcis*.. 128
AA.	All color markings yellow.	var. *affinis*.. 128

Erythroneura basilaris Say.

Tettigonia basilaris Say. Journ. Acad. Nat. Sc., Phila., IV, p. 344; 1825. Reprint Compl. Writings II, p. 260; 1859.

Typhlocyba comes var. *basilaris* Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 760; 1898.

Color Pattern. Ground color yellowish-white. Vertex and anterior part of pronotum sometimes marked with light red, but frequently clouded over with a dark smoky band which involves the posterior part of pronotum, scutellum and basal half of tegmina. Inner basal angle of clavus whitish; apical half of tegmina marked with yellow or red. Cross-veins red; a black spot in base of cell M_4.

Inner Male Genitalia. (Pl. V.)

Host Plants. This species has been found breeding on American elm and wild plum.

Distribution. Ontario, New York, Connecticut, Virginia, Tennessee, Ohio, Illinois, Wisconsin, Iowa, Nebraska, Kansas.

Erythroneura basilaris var. *dulcis* McAtee.

Erythroneura basilaris var. *dulcis* McAtee. Trans. Am. Ent. Soc., XLVI, p. 296; 1920.

Color Pattern. "Tegminal band pinkish, tegminal spots yellow, markings on scutellum and anterior parts transculent to livid." (McAtee.)

Distribution. Maryland, Kansas.

Erythroneura basilaris var. *affinis* Fitch.

Erythroneura affinis Fitch. N. Y. St. Cab. Nat. Hist., p. 63; 1851. Lintner, Reprint, p. 403; 1893. (New York.)

Color Pattern. "Tegminal band yellowish brown, other color markings yellow, those on anterior part of pronotum and on vertex sometimes obsolete." (McAtee.)

Distribution. Kansas.

Erythroneura ligata McAtee may be easily distinguished by the "broad zigzag scarlet vittæ on tegmina, which inclose two pale saddle-spots, a smaller one on inner anterior portions of clavi and a larger one, embracing apical third of clavi and adjacent parts of corium." (McAtee.)

KEY TO VARIETIES OF *LIGATA.*

A. Head and pronotum with faint yellow markings, tip of clavus unmarked. PAGE
var. *ligata*.. 128

AA. Head and pronotum with red markings, tip of clavus with a red spot.
var. *allecta*.. 129

Erythroneura ligata McAtee.

Erythroneura ligata McAtee. Trans. Am. Ent. Soc., XLVI, p. 301; 1920.

Color Pattern. "Ground color of scutellum and anterior parts ivory white, of tegmina whitish-hyaline. Vertex with transculent to yellow spots, arranged as in *E. hartii*, three on transition from vertex to front, and four between eyes, of which the central pair are larger and may be connected with lateral spots or with posterior margin of vertex. Pronotum with two broad

lateral, and two narrower discal vittæ, which may be broken into smaller anterior and larger posterior spots, transculent to yellow. Scutellum with yellow basal triangles outlined by scarlet, apex yellow. Tegmina with broad zigzag scarlet vittæ which inclose two pale saddle-spots, a smaller on inner anterior portions of clavi and a larger embracing posterior third of clavi and adjacent parts of corium; the anterior part of each vitta is confined to clavus, and the posterior part, after angling laterally to costal plaque, returns to radial margin and ends abruptly at the cross-veins, a lateral prolongation following them to costal margin; a faint yellow dot at base of corium, usually an oblique yellow streak on anterior margin of costal plaque, plaque itself whitish, a black or bluish dot at its posterior margin, a dusky cloud in base of fourth apical cell, apical cells in general slightly fumose." (McAtee.)

Inner Male Genitalia. (Pl. V.)

Distribution. District of Columbia, Illinois, Kansas.

Erythroneura ligata var. *allecta* McAtee.

Erythroneura ligata var. *allecta* McAtee. Trans. Am. Ent. Soc., XLVI, p. 302; 1920.

Color Pattern. "Like typical variety, except that yellow markings of pronotum, vertex and costal margin are replaced by red; apex of scutellum and a spot near tip of clavus also red; touches of red on pleura and face in some specimens." (W. L. McAtee.)

Distribution. District of Columbia, Illinois, Kansas.

THE *COMES* GROUP.

This is the largest and most intricate group in the genus. It may be separated from the preceding by the square base of cell M_4 (Pl. I); and the presence of a black spot or cloud in the apex of cell R_3 and base of cell M_4. The pygofer hooks of the inner male genitalia are very characteristic of the group. Each forms a U-shaped process lying on its side with caudally directed arms (Pl. I), the lower arm always being the longer. There is a considerable range of variation in these hooks within the group, but the characteristic U-shape is retained throughout.

In color pattern there is a very wide range, from an almost solid velvety-black, as in *infuscata*, to a milky white as in *lacta*, and from cross-bands to oblique longitudinal vittæ. There occurs, however, with more or less distinctness throughout the group, an inverted V- or U-shaped mark upon the vertex and a Y-shaped mark upon the pronotum. The group includes twenty-seven species and fourteen varieties.

KEY TO SPECIES. PAGE

A. Chief color markings of upper surface one or more transverse bands.
  B. Bands three in number.
    C. Anterior crossband covering bases of tegmina; bands broad.
      D. Vertex and pronotum narrower, the latter not broader than long, pronotum uniformly deep maroon. *rubranotum*.. 137
      DD. Vertex and pronotum broader, the latter about one and one-half times broader than long, pronotum red to dusky, frequently with whitish marks on anterior margin. *vitis*.. 143
    CC. Anterior crossband not covering bases of tegmina; bands narrower. *tricincta*.. 139
  BB. With one band. This band is reddish to dusky and extends over basal half of tegmina, involving tip of scutellum, most of clavi, and area between clavi and costal plaques. *amanda*.. 137
AA. Chief color markings of upper surface otherwise.
  B. Upper surface almost completely smoky-brown to black. *infuscata*.. 136
  BB. Upper surface not as above.
    C. A dark angulate stripe the length of dorsum, pointed on vertex and broad over tegmina. *aclys*.. 135
    CC. Coloration otherwise.
      D. Tegminal markings angulate vittæ, continuous from humeri to crossveins.
        E. Tip of clavus spotted.
          F. Vitta extending from humerus to costal plaque, thence to crossveins; upper surface from scutellum to crossveins occupied by a pale diamond-shaped area with red to yellow spots. *cancellata*.. 141
          FF. Vitta extending from humerus on to the clavus, leaving a pale area anterior to the costal plaque.
            G. A single median vitta on vertex; median vitta on pronotum weakly bifurcate anteriorly.
              H. Larger species, 3 mm. in length; vittæ bright red; a large yellow spot around tip of clavus; ground color yellowish. *fraxa*.. 147
              HH. Smaller species, 2.5 mm. in length; vittæ sanguineous; a red spot at tip of clavus; ground color whittish diffused with red. *kanwakæ*.. 143
            GG. Two vittæ, if any, forming an inverted V-shaped mark on vertex; median vitta on pronotum more strongly bifurcate anteriorly.
              H. Vittæ on tegmina and sides of pronotum broader and sanguineous to dusky.
                I. Tip of clavus lighter than main tegminal vittæ; sides of pronotum heavily marked; main tegminal vittæ of nearly uniform width throughout. *ziczac*.. 135
                II. Tip of clavus about the same color as main tegminal vittæ; sides of pronotum lightly marked; main tegminal vittæ varying in width. *portea*.. 142
              HH. Vittæ on tegmina and sides of pronotum narrower and bright red.
                I. Scutellum marked with two conspicuous black spots at base. *coloradensis*.. 134
                II. Scutellum without black spots at base.
                  J. Mesosternum glossy-black; eyes grey to fuscous. *vitifex*.. 134
                  JJ. Mesosternum yellowish-white; eyes black. *beameri*.. 141

EE. Tip of clavus unmarked. PAGE
F. Tegminal markings narrower, bright red, and of nearly uniform width throughout; an unusually short and slender species. *acuticephala*.. 146
FF. Tegminal markings broader, pale red to sanguineous, outlined by brighter red, greatly broadened between the costal plaques. *elegans*.. 137
DD. Tegminal markings otherwise, not continuous from humeri to crossveins.
E. Chief tegminal markings consisting of an elongate vitta in basal half of clavus and spot at tip, an oblique streak in base of corium, an angulate vitta more or less completely bordering inner margins of costal plaque.
F. Upper surface of abdomen blackish and showing through tegmina.
G. Vertex yellowish-white with a median inverted V- or U-shaped vitta of red or yellow.
H. All vittæ on upper surface very broad and bright red, giving the insect a characteristic red appearance. *rubra*.. 135
HH. Upper surface not so marked.
I. Clavus bearing a large black spot near middle. *cherokee*.. 142
II. Clavus without black spot. *omaska*.. 146
GG. Vertex red, with eight yellowish-white spots which are sometimes fused to make four longitudinal vittæ.
H. Vertex broader and more acutely pointed; tegmina semihyaline; dorsal markings bright red. *corni*.. 138
HH. Vertex narrower and more rounded at apex; tegmina white; dorsal markings pale red. *ontari*.. 136
FF. Upper surface of abdomen yellowish-white.
G. Tegminal vittæ red or yellow, distinct; crossveins about same color as vittæ. *comes*.. 131
GG. Tegminal vittæ orange, without definite margins, the whitish ground color being diffused with pale orange; crossveins brilliant crimson. *aza*.. 138
EE. Chief tegminal markings otherwise.
F. Middle of clavus with a conspicuous triangular area; base and tip frequently bearing a small spot.
G. Black spot in triangular area of clavus.
H. Ground color of tegmina milky-white; markings red. *scripta*.. 147
HH. Ground color greenish-brown on anterior half, remainder whitish; markings brownish to red. *tudella*.. 138
GG. Without such black spot. *lacta*.. 146
FF. Middle of clavus without triangular area; tegminal markings made up of dots situated chiefly in the region of the longitudinal veins and at the humeri. *irrorata*.. 143

Erythroneura comes Say, one of the most commonly occurring species, probably is listed in every collection of *Erythroneura*. Several species, however, somewhat resemble *comes* in color pattern and may be very easily confused with it; and it might not be unacceptable to sound a note of warning in this respect. I have examined

collections labeled *comes* which contained as many as eight different species. The latitude taken in classifying members of this genus is almost certainly due to the prevalent idea that extensive variations exist within the genus. It is now demonstrated that there is considerably less variation than has been supposed, and that forms having even a slight difference in color pattern may be found, when the inner male genitalia are examined, to be distinct species.

As many as twenty varieties of *comes* have been described. McAtee (1920)[6] raised five of these varieties to specific rank, on the characters of wing venation and color pattern. This was confirmed in the same year by Lawson[7] in his study of the inner male genitalia. The number of varieties is now reduced to four, and possibly less, as I have not been able to examine the inner male genitalia of one of the forms left as a variety, namely, *venusta* McAtee, which probably should be raised to specific rank.

KEY TO VARIETIES OF *COMES*.

		PAGE
A.	Scutellum and clavi lacking black markings.	
B.	Ground color whitish-hyaline or yellowish-white. *comes*. .	132
BB.	Ground color milky-white, nearly opaque. var. *delicata*. .	133
AA.	Scutellum or clavi, or both, with dark markings.	
B.	Tegminal vittæ dusky, interrupted behind costal plaque by a broad transverse whitish-hyaline area; pronotum and basal triangles of scutellum ruby-red, apex of scutellum narrowly margined with black. var. *venusta*. .	133
BB.	Tegminal vittæ or spots red to yellow.	
C.	Scutellum without dark markings; spots on inner margins of clavi black. var. *octonotata*. .	133
CC.	Scutellum with dark markings consisting of a median vitta or a spot at apex. var. *compta*. .	133

Erythroneura comes Say.

Tettigonia comes Say. Jour. Acad. Nat. Sci., Phila., IV, p. 343; 1825. Compl. Writ. II, p. 259; 1859.

Color Pattern. Ground color of vertex, pronotum and scutellum yellowish-white, of tegmina white to semi-hyaline, and marked with red or yellow as follows: Vertex, an inverted U-shaped median vitta and a small ring along the posterior margin opposite each eye. These marks are sometimes rather irregular in outline; however, they tend to enclose three yellowish-white areas, the middle one of which is twice as long as the lateral ones. Pronotum, a Y-shaped median vitta and a lateral elongated area behind each eye. Scutellum, a spot or outline of one in each basal angle and a small dot at tip. Tegmina, an elongate vitta, abruptly swollen at tip and projecting inward to the tegminal suture, in basal half of clavus, a spot at apex; on corium, an oblique streak near base, a line arising before costal plaque, proceeding inward along the inner margin of plaque to its apex and thence to base of cell M_4. Midway along the inner margin of costal plaque there is usually a short, broad extension which approaches to, but does not connect with, the claval vitta. The

[6] W. L. McAtee: Trans. Am. Ent. Soc., XLVI, 267-322; 1920.
[7] Lawson, P. B.: Kans. Univ. Sc. Bul., vol. XII, No. 1; 1920.

corial and claval vittæ therefore do not form a continuous line from humerus to cross veins. Cross veins and adjacent parts of longitudinal veins same color as dorsal markings. Costal plaque opaque-white, but frequently scaled off; an oblique black streak at apex; a black spot in apex of cell R_3 and base of cell M_4.

Inner Male Genitalia. (Pl. VI.)

Host Plants. Grape, cultivated and wild. While it is a very general feeder in the spring and late autumn, in the absence of grape foliage, it is not known to breed upon any plant but the grape.

Distribution. This is a wide-spread species and occurs practically everywhere the grape is grown.

Erythroneura comes var. *octonotata* Walsh.

Erythroneura octonotata Walsh. The Prairie Farmer (Illinois), p. 149; 1862.

Color Pattern. Same as *comes* and having in addition a black spot on the middle of the inner margin of the clavus. The color markings are sometimes yellow instead of red.

Host Plants. Grape.

Distribution. Ontario, New York, Maryland, Mississippi, Michigan, Illinois, Kansas, California.

Erythroneura comes var. *compta* McAtee.

Erythroneura comes var. *compta* McAtee. Trans. Am. Ent. Soc., XLVI, 267-322; 1920.

Color Pattern. Same as *comes,* but having in addition a black spot on the middle of the inner margin of the clavus and another near tip of scutellum.

Host Plants. Grape.

Distribution. Ontario, Maryland, Virginia, Alabama, Mississippi, Michigan, Indiana, Illinois, Kansas.

Erythroneura comes var. *delicata* McAtee.

Erythroneura comes var. *delicata* McAtee. Trans. Am. Ent. Soc., XLVI, p. 317; 1920.

Color Pattern. "Ground color opaque milky white, with red or yellow color markings of the *comes* type, the spots usually small and well separated." (McAtee.)

Distribution. Maryland.

Erythroneura comes var. *venusta* McAtee.

Erythroneura comes var. *venusta* McAtee. Trans. Am. Ent. Soc., XLVI, p. 319; 1920.

Color Pattern. "Vertex chiefly dusky, narrow anterior border and median streak pale yellow; thorax and basal triangles of scutellum deep ruby-red, apex black; broad dusky vitta on tegmen occupying anterior two-thirds of clavus and an oblique area on corium from clavus to black marking on costal plaque; tegmen from posterior edge of this band to cross veins whitish-hyaline; apical cells chiefly dusky; under surface pale yellow." (McAtee.)

Distribution. Maryland.

Erythroneura vitifex Fitch.

Erythroneura vitifex Fitch. Insects Infesting Fruit Trees. Trans. N. Y. State Agr. Soc., XXI, p. 392; 1856.

Color Pattern. General ground color creamy-white and marked with red as follows: An inverted V-shaped vitta upon vertex; a Y-shaped median and two lateral vittæ upon pronotum; scutellum with a red spot in each angle; on tegmina a zigzag vitta extending from humerus midway along clavus, thence over the costal plaque and base of cell M_4, tip of clavus red; cross-veins red; apical cells dusky; an oblique black streak in apex of costal plaque; a black spot in base of cell M_4.

Frequently this species is marked with yellow instead of red on anterior parts.

Host Plants. Grape.

Inner Male Genitalia. The genitalia differ markedly from those of *comes* in several particulars. (Pl. VI.)

Distribution. Ontario, New York, Virginia, Michigan, Indiana, Illinois, Wisconsin, Iowa, Kansas, Texas, New Mexico.

This species is sometimes confused with *comes*, which, however, it does not closely resemble, as its tegminal color markings consist of a continuous vitta from humerus to cross veins, while those of *comes* are interrupted. The position of this form within the genus has been a changeable one, being regarded at times as synonymous with *comes* or as one of its varieties. However, its inner male genitalia prove it to be a distinct species. There is a close resemblance in color pattern between *vitifex* and *cherokee*. These two species, which show outstanding differences in their inner male genitalia, may be separated readily by the fact that in the former the mesosternum (visible between the first and second pairs of legs) is glossy-black while in the latter it is yellowish-white.

Erythroneura coloradensis Gillette.

Typhlocyba vitifex var. *coloradensis* Gillette. Colo. Agr. Exp. Sta., Bul. 19, p. 16; 1892.

Color Pattern. Resembles *vitifex*, and has in addition two black spots in the base of the scutellum.

Inner Male Genitalia. This is quite characteristic, and differs greatly from *comes* and *vitifex*. (Pl. VI.)

Host Plants. Grape.

Distribution. Illinois, Nebraska, Kansas, Colorado, New Mexico, California.

This form, which has been regarded as a variety of both *comes* and *vitifex*, is here raised to specific rank.

Erythroneura ziczac Walsh.

Erythroneura ziczac Walsh. The Prairie Farmer (Illinois), 10, No. 10, p. 149; 1862.
Typhlocyba comes var. *ziczac* Gillette. Proc. U. S. Nat. Mus., p. 761; 1898.

Color Pattern. Vertex yellow, pronotum yellow on anterior half and darker behind, a broad brown mark on lateral margins; scutellum yellow with a brown spot in basal angles; tegmina, a broad brown vitta extending from humerus to middle of clavus, then to costal plaque and to base of cell M_4; tip of clavus yellow; an oblique black streak at apex of costal plaque; a black dot in apex of cell R_3 and a smoky area in base of cell M_4; apical cells dusky.

Inner Male Genitalia. (Pl. VII.)

Host Plants. Grape, Virginia creeper, Boston ivy (*Ampelopsis* spp.).

Distribution. Ontario, Maryland, Michigan, Illinois, Mississippi, Iowa, Nebraska, Kansas, Colorado, California.

This species appears to have an especial preference for *Ampelopsis*, and because of the smoothness of the under surface of the foliage the eggs laid under the epidermis may be seen readily, frequently surrounded by a bluish-gray deposit. They are commonly laid side by side in groups of from three to eight. I have never seen *comes* lay eggs other than singly and well separated.

This form is here again raised to specific rank.

Erythroneura rubra Gillette.

Typhlocyba comes var. *rubra* Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 764; 1898.

Color Pattern. Similar in formation to *comes*, but the vittæ are very much broader and of a bright red or scarlet color, sometimes outlined in deep crimson.

Inner Male Genitalia. (Pl. VI.)

Host Plants. Grape.

Distribution. Maryland, Virginia, South Carolina, Tennessee, Illinois, Iowa, Kansas.

This form is here raised to specific rank.

Erythroneura aclys McAtee.

Erythroneura aclys McAtee. Trans. Am. Ent. Soc., XLVI, p. 290; 1920.

Color Pattern. "Consisting of a broad brown to black stripe extending whole length of insect, margined by pale yellow. The dorsal stripe nearly black on scutellum and anterior parts sharply cut off from a pale yellow margin about the width of eye; on tegmina the stripe is smoky brown, greatly expanded and irregularly set off from the pale yellow costal margin, percurrent to second apical cell, which is about the width of costal plaque; the latter has a slight opaque whitish coating, is margined interiorly and anteriorly by reddish, and underlaid posteriorly by blackish; there are touches of red upon apex of clavus and first cross vein and more or less hyaline spots at extremities of apical third of clavus, on the corium nearby and in middle of fourth apical cell. The legs, pleura and face, vary from flesh color to pale yellow, and the

venter is pale yellow with a median series of slaty spots, the posterior ones extending entirely across last abdominal segment and across base of genitalia; dorsum slaty." (McAtee.)

Inner Male Genitalia. (Pl. VI.)

Host Plants. Redbud (*Cercis canadensis*).[8]

Distribution. Connecticut, New Jersey, Maryland, South Carolina, Tennessee, Illinois, Missouri, Kansas, Nebraska.

This species is generally mistaken for *E. infuscata* due to the fact that Gillette's description of the latter is that of *E. aclys*, while his type in the United States National Museum is that of *infuscata*.

Erythroneura infuscata Gillette.

Erythroneura comes var. *infuscata* Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 764; 1898.

Color Pattern. The following description by W. L. McAtee was made from the type in the United States National Museum: "Smoky brown to black above, in some cases underlaid by deep dull red, a little paler at rondure of vertex, especially on sides, pronotum sometimes faintly paler on disc, and scutellum sometimes with a pale median vitta; tegmina with more or less hyaline spots at base, middle and apex of inner margin of clavus, middle of radial margin of fourth apical cell and on costal margin of second apical cell; costal plaque opaque whitish, tinged with red, an almost equal area of dull red just posterior, and costal margin and to some extent the cross veins penciled with dull red. Face and legs pale yellow, more or less tinged or marked with red, remainder of under surface slaty, abdominal incisures, etc., sometimes pale yellow."

Inner Male Genitalia. Pl. VIII.

Distribution. Maryland, Mississippi, Illinois, Kansas.

The original description of this form by Gillette in the work noted above is not the description of his type in the United States National Museum, but that of another species, namely, *aclys* McAtee. A certain amount of confusion has arisen because of this discrepancy.

Erythroneura ontari Robinson.

Erythroneura ontari Robinson. Can. Ent., vol. LVI, No. 3, p. 60; 1924.

Color Pattern. Vertex yellow, chief red markings being a median and two lateral thin streaks. Ground color of pronotum, scutellum and tegmina greyish-white faintly marked with red, as follows: Pronotum with heavy Y-shaped median vitta, and a streak on each side behind the eyes; scutellum with a spot in basal angles and at tip. Tegmina marked as follows: A broad vitta on clavus in touch with basal half of claval suture and barbed at caudal end, and a spot in apical third; corium with a broad stripe, with five lateral extensions running the entire length of cubitus, the three inner ones extending toward the claval suture at its base, middle and tip respectively, and the two

8. H. B. Weiss and E. West: Ent. News, vol. XXXV, No. 4, p. 129; 1924.

outer ones touching the anterior and posterior parts respectively of the costal plaque; red streaks between costal plaque and red cross veins; base and tips of cells smoky.

Inner Male Genitalia. This species has a long and curved lower arm to the pygofer hooks, a long slender œdagus, and a truncate, abruptly curved apex to the styles. (Pl. VIII.)

Distribution. Ontario, Canada.

Erythroneura amanda McAtee.

Erythroneura comes var. *Amanda* McAtee. Trans. Am. Ent. Soc., XLVI, p. 319; 1920.

Color Pattern. "Apical third, or at least the sides of scutellum, apically, dusky to black; anterior two-thirds of clavus and a band between clavus and costal plaque bright red; subsidiary markings of tegmen tending to be red anteriorly and yellow posteriorly; markings of head and pronotum yellow." (McAtee.)

Inner Male Genitalia. The apical process of the œdagus is divided into four spines. This feature alone is sufficient to give this form specific rank. (Pl. VII.)

Host Plants. Grape.

Distribution. Missouri, Kansas.

Erythroneura rubranotum Robinson.

Erythroneura rubranotum Robinson. Can. Ent., vol. LVI, No. 3, p. 60; 1924.

Color Pattern. Vertex ivory, with apex and two small spots on base faintly red. Pronotum and basal half of scutellum deep maroon; tip of scutellum white. Tegmina, ground color light yellow, red band across base, a wide red band with darker markings occupying apical half, excepting tip, of clavus and extending over corium to an elongate brown area in the region of costal plaque; cross veins red, apical cells smoky.

Inner Male Genitalia. (Pl. VI.)

Distribution. Kansas.

Erythroneura elegans McAtee.

Erythroneura comes var. *elegans* McAtee. Trans. Am. Ent. Soc., XLVI, p. 315; 1920.

Color Pattern. "This variety (species) differs from *vitifex* in having broader tegminal vittæ, pale bluish-red to dusky within, brighter margined, which are in touch for a greater distance along the commissure (at middle of clavus); the posterior pale spot inclosed by them is less extensive and the tips of the clavi are not marked with red." (W. L. McAtee.)

Inner Male Genitalia. (Pl. VIII.)

Host Plants. Grape; Boston ivy (*Ampelopsis* spp.).

Distribution. Ontario, Pennsylvania, Maryland, Mississippi, Arkansas, Indiana, Illinois, Iowa, Nebraska, Kansas, Colorado.

This species differs considerably from *comes* in color pattern, habits, and particularly inner male genitalia; and is therefore raised to specific rank.

Erythroneura corni Robinson.

Erythroneura corni Robinson. Can. Ent., vol. LVI, No. 3, p. 60; 1924.

Color Pattern. Vertex brownish-red with two transverse rows of four white spots each, the middle spots connected to form longitudinal vittæ; posterior half often of lighter color. Pronotum, anterior margin yellow, a large brown discal area, a lateral red spot behind each eye; two smoky-grey spots on posterior margin. Scutellum brown, with faint median and transverse line yellow. Tegmina hyaline, with the following pattern in red: On clavus, a vitta in touch with claval suture from the base about one-half the length of the latter, and a spot covering apical third; on corium, a vitta extending from near base of wing to costal plaque, thence inwardly half way around the latter, where it becomes enlarged and almost touches claval suture, then narrows again and continues to the posterior end of the costal plaque, where it forms a "Y" with heavy base, one arm extending to the tip of clavus and the other along cubitus to the red cross veins. Apical cells smoky, except middle of cells R_3 and M_4. An oblique dark line in apex of costal plaque. The blackish abdomen shows through the tegmina and gives the insect a dark appearance.

Inner Male Genitalia. This species has a long and upward curving lower arm to the pygofer hooks; and a long slender œdagus. (Pl. VII.)

Host Plants. Dogwood (*Cornus* spp.).

Distribution. Kansas, Nebraska.

Erythroneura aza Robinson.

Erythroneura aza Robinson. Can. Ent., vol. LVI, No. 12, p. 291; 1924.

Color Pattern. Ground color of vertex, pronotum and scutellum ivory, of tegmina white, with the following marks in orange. Vertex, a narrow median inverted U-shaped vitta; pronotum, a broad median Y-shaped vitta and a broad lateral vitta behind each eye; scutellum, a broad spot in each basal angle and a small spot at tip. Tegmina, lightly washed with orange throughout, except at inner basal angle of clavus and in base of corium, where the orange is deepened to nearly red. Cross veins and adjacent parts of longitudinal veins crimson. A black streak caudad of costal plaque; apical cells whitish at base, remainder dusky, especially near the base. A black spot in apex of cell R_3 and base of cell M_4.

Inner Male Genitalia. (Pl. VII.)

Distribution. Kansas.

Erythroneura tudella Robinson.

Erythroneura tudella Robinson. Can. Ent., vol. LVI, No. 12, p. 291; 1924.

Color Pattern. Vertex brownish, eyes black; pronotum fulvous on anterior margin, remainder greenish-yellow; scutellum yellow. Tegmina, ground color of basal third greenish-yellow, of remainder white, and marked as follows: In middle of clavus a brownish-red somewhat triangular area with a black spot in the angle touching the tegminal suture; a brownish-red line arising before the costal plaque and bordering its inner margin, the line then broadens, becomes orange in color and proceeds to the base of cell M_4. Costal plaque

opaque-white with an oblique black streak at its apex; apical cells smoky; a black spot in apex of cell R_3 and base of cell M_4.

Distribution. Kansas.

Erythroneura tricincta Fitch is one of the most common and easily recognized species, and is recorded as being frequently injurious to grape. Six varieties occur, and may be separated by the following key.

KEY TO VARIETIES OF *TRICINCTA*.

A. Anterior cross-band involving base of scutellum.
  B. Anterior crossband extending along sides of pronotum, leaving disc uncolored; middle crossband sanguineous to dusky, black on costa. var. *calycula*.. 140
  BB. Anterior crossband covering all of pronotum except a small part of anterior margin.
    C. Crossbands sanguineous to black. *tricincta*.. 139
    CC. Crossbands bright red.
      D. Longitudinal color streaks yellow. var. *diva*.. 139
      DD. Longitudinal color streaks bright red. var. *rubravitta*.. 140
AA. Anterior crossband not covering base of scutellum.
  B. Anterior crossband sanguineous to dusky, covering most of pronotum; middle crossband sanguineous to bright red, black on costa; posterior crossband yellow-brown on apical half. var. *integra*.. 140
  BB. Anterior crossband dusky to black, profoundly emarginate anteriorly, leaving a large part of disc of pronotum uncolored.
    C. Crossband continuous across posterior margin of pronotum. var. *cymbium*.. 140
    CC. Crossband (?) broadly interrupted, covering only sides of pronotum. var. *disjuncta*.. 140

Erythroneura tricincta Fitch.

Erythroneura tricincta Fitch. N. Y. State Cab. Nat. Hist., IX, p. 63; 1851.

Color Pattern. The anterior crossband covers all of pronotum except a small area on anterior margin, and basal half of scutellum. The longitudinal color streaks upon the yellowish-white background between the crossbands may be either yellow or red.

Inner Male Genitalia. (Pl. VI.)

Host Plants. Grape, Boston ivy (*Ampelopsis* spp.).

Distribution. Ontario, New York, Vermont, New Hampshire, Pennsylvania, Maryland, Virginia, North Carolina, South Carolina, Tennessee, Ohio, Michigan, Indiana, Mississippi, Illinois, Wisconsin, Iowa, Missouri, Texas, Kansas, Nebraska, Colorado.

Erythroneura tricincta var. *diva* McAtee.

Erythroneura tricincta var. *diva* McAtee. Trans. Am. Ent. Soc., XLVI, p. 308; 1920.

Color Pattern. "Crossbands one and two chiefly bright red; crossband one covering most of pronotum and base of scutellum, where its color is sometimes brownish-yellow instead of red; crossband two, bright red to costal plaque which is chiefly blackish, more or less overlaid by opaque white; longitudinal color markings sulphur yellow." (McAtee.)

Distribution. Maryland, Virginia, Kansas.

Erythroneura tricincta var. *calycula* McAtee.

Erythroneura tricincta var. *calycula* McAtee. Trans. Am. Ent. Soc., XLVI, p. 308; p. 1920.

Color Pattern. "Ground color ivory, a broad U-shaped dusky to black band on base of scutellum and sides of pronotum, the often concolorous eyes extending in on vertex; crossband two sanguineous to dusky, black on costa; longitudinal color streaks red." (McAtee.)

Distribution. Maryland, Illinois, Kansas, Nebraska.

Erythroneura tricincta var. *integra* McAtee.

Erythroneura tricincta var. *integra* McAtee. Trans. Am. Ent. Soc., XLVI, p. 309; 1920.

Color Pattern. "Crossband one, sanguineous to dusky, confined to pronotum, most of which it covers; crossband two bright red to sanguineous, bluish to black on costa; longitudinal color markings yellow or red." (McAtee.)

Distribution. Maryland.

Erythroneura tricincta var. *cymbium* McAtee.

Erythroneura tricincta var. *cymbium* McAtee. Trans. Am. Ent. Soc., XLVI, p. 310; 1920.

Color Pattern. "Crossband one, dusky to black confined to pronotum, profoundly emarginate anteriorly, leaving a large part of disc uncolored; crossband two sanguineous to red, bluish to black on costa; longitudinal color streaks yellow." (McAtee.)

Distribution. Ontario, Pennsylvania, Michigan, Nebraska, Kansas, Texas.

Erythroneura tricincta var. *disjuncta* McAtee.

Erythroneura tricincta var. *disjuncta* McAtee. Trans. Am. Ent. Soc., XLVI, p. 310; 1920.

Color Pattern. "Like the last, but cross-band (if it may be so called) one, broadly interrupted in the middle, covering only sides of pronotum." (McAtee).

Distribution. Pennsylvania, Kansas.

Erythroneura tricincta var. *rubravitta* Robinson.

Erythroneura tricincta var. *rubravitta* Robinson. Can. Ent., vol. LVI, No. 7, p. 156; 1924.

Color Pattern. Vertex yellow, with median dashes of red. Pronotum maroon, except a small yellow spot in middle of anterior margin. Scutellum deep maroon in basal angles and light red or yellow at tip. Tegmina, ground color yellow with the following marks in red: A broad band across middle ending in the large black area in each costal plaque. In this band the color is pale within and with dark margins. A broad vitta lies in base of clavus and there is a faint spot at tip. There is also a spot in base of corium. Longitudinal veins, especially cubitus, overlaid with red. Cross veins blackish; apical cells, excepting base, smoky to black.

Distribution. Kansas.

Erythroneura cancellata McAtee.

Erythroneura comes var. *cancellata* McAtee. Trans. Am. Ent. Soc., XLVI, p. 320; 1920.

Color Pattern. "Vertex and pronotum dusky sanguineous, the usual vittæ barely distinguishable by their brighter red color; scutellum sanguineous, except broad median pale vitta, and bright red tip; on each tegmen a broad sanguineous vitta from humerus to costal plaque and from costal plaque to cross veins near radial margin; these vittæ inclose (with pale areas on scutellum) a large pale diamond-shaped saddle-spot extending from base of scutellum to cross veins, which is ornamented by bright red markings as follows: Tip of scutellum and of clavus, a V-shaped marking on middle of each clavus, connected by a short oblique streak to longitudinal sanguineous vitta near posterior end of costal plaque. Costal plaque pale yellow, an oblique black streak posteriorly, more or less overlaid by opaque white; costa between plaque and cross veins pale yellow, a ramose red marking on cross veins; apical cells dusky except extreme bases of all, and the central interior of two and four; a little black at base of four and at apices of two and four. Pale yellow below, face with touches of red, and genitalia more or less livid to slaty." (McAtee.)

Inner Male Genitalia. (Pl. VII.)

Distribution. Maryland, Kansas.

This form is here raised to specific rank.

Erythroneura beameri Robinson.

Erythroneura beameri Robinson. Can. Ent., vol. LVI, No. 3, p. 61; 1924.

Color Pattern. General ground color creamy-white with the following red markings: Vertex, two posteriorly diverging vittæ making an inverted V-shaped mark; pronotum, a broad Y-shaped vitta with arms and base of about equal width and length, a vitta on each lateral margin; scutellum, a solid spot or outline of one in each basal angle and a spot at the tip; tegmina, on clavus a vitta in touch with basal half of claval suture with a caudal barb which projects forward and inward to meet the tegminal suture, tip of clavus red; on corium an oblique line arising near the humeral angle and meeting the middle of the claval vitta, another which borders front and inner margins of the costal plaque and then proceeds to the base of cell M_4. Cross veins red; cells smoky. An oblique dark mark in the apex of costal plaque; a black dot in the apex of cell R_3 and base of cell M_4.

Inner Male Genitalia. (Pl. VI.)

Distribution. Kansas.

This species somewhat closely resembles *vitifex* in color pattern, but may be separated by the fact that the mesosternum is yellowish-white instead of black. The great difference in the inner male genitalia of these two forms precludes any possibility of their being the same species.

Erythroneura portea Robinson.

Erythroneura portea Robinson. Can. Ent., vol. LVI, No. 7, p. 154; 1924.

Color Pattern. General ground color yellowish-white, with the following markings: Vertex, an inverted V-shaped reddish vitta. Pronotum, a slaty-brown area on disk with two reddish arms projecting forward to meet the vitta on vertex; a lateral vitta behind each eye. Scutellum, a yellowish spot outlined in red in each basal angle, and yellow spot at tip. Tegmina, a reddish vitta arising in base of clavus, soon broadening and proceeding across corium to costal plaque, where it becomes as wide as length of plaque, then proceeding inward and dividing into two arms, one of which touches tip of clavus and the other the base of cell M_4. This vitta is sometimes paler within and margined in dark red; sometimes bluish within dark red margin; tip of clavus is pale red. Costal plaque yellowish-white with an oblique black or blue streak at apex. Cross veins and adjacent parts of longitudinal veins crimson. Apical cells smoky, except part adjoining cross-veins.

Inner Male Genitalia. (Pl. VII.)

Host Plants. Grape.

Distribution. Ontario, Kansas.

Erythroneura cherokee Robinson.

Erythroneura cherokee Robinson. Can. Ent., vol. LVI, No. 7, p. 154; 1924.

Color Pattern. Vertex yellow, with a median U-shaped vitta and two lateral streaks reddish. Pronotum yellow on anterior margin and slaty behind; on disk and close to posterior margin a brownish-red area with two hornlike prolongations projecting forward to meet the median vitta on vertex, a wide reddish lateral vitta behind each eye. Scutellum, a yellow spot outlined in brown in each basal angle, a yellow spot at tip, a white inverted T-shaped vitta on disk. Tegmina hyaline, with the following red markings: Basal two-thirds of clavus occupied by a hatchet-shaped vitta, which contains a black spot in its broad end; a spot at tip. On corium, an oblique streak at base, a streak bordering front of white costal plaque and broadening at middle of the inner margin until it touches the claval suture, proceeding backward in a narrowing line to the blue streak at apex of costal plaque, then continuing backward to the base of cell M_4. Cross veins and adjacent parts of longitudinal veins crimson; apical veins white; apical cells hyaline bordering cross veins and followed by a dark smoky area, remainder of cells light smoky; a black spot in the apex of cell R_3 and base of cell M_4.

Inner Male Genitalia. The apical process of the œdagus is seen to be divided into two slender spines, when viewed laterally, the inner one being the longer. Each of these spines is in turn divided, and when viewed dorsally the œdagus appears to bear four apical spines. The apex of the styles bears a long slender tooth. (Pl. VIII.)

Distribution. Nebraska, Kansas.

Erythroneura kanwakae Robinson.

Erythroneura kanwakae Robinson. Can. Ent., vol. LVI, No. 12, p. 292; 1924.

Color Pattern. Vertex ivory, with two very narrow vittæ of red which lie closely together and frequently are fused for the greater part of their length. Pronotum ivory in the anterior half, the remainder yellow tinged with grey; a Y-shaped vitta on disk and two lateral lines red. Scutellum yellowish-white, with a red spot in each angle. Tegmina white and marked with red, as follows: On clavus, a vitta in touch with basal two-thirds of claval suture and a spot at tip. On corium, a spot at base, a fine line arising in front of costal plaque, soon broadening and proceeding around inner margin, giving off a short, broad extension to the claval suture and touching claval vitta, then directed inward to the base of cell M_4. The corial vittæ are sometimes pale red to dusky within and brightly margined in red; whitish ground color of tegmina frequently diffused with red; a smoky band extends obliquely over apical cells from base of M_4 to apex of R_3. This is a slender species with a long narrow vertex.

Inner Male Genitalia. The pygofer hooks which are characteristically U-shaped in this group are, in *kanwaka*, somewhat modified by bearing between the two arms an intermediate spine. In this species the pygofer hooks are also especially large. The dorsal part of the œdagus bears two acute and forward projecting teeth. The apices of the styles are abruptly swollen and truncate. (Pl. VIII.)

Distribution. Nebraska, Kansas.

Erythroneura irrorata Robinson.

Erythroneura irrorata Robinson. Can. Ent., vol. LVI, No. 7, p. 154; 1924.

Color Pattern. Vertex red, with a median and two lateral streaks of yellow. Pronotum, anterior part red, with three yellow spots, remainder slaty-grey. Scutellum orange, with darker spots in basal angles and tip. Tegmina hyaline, irrorate with red in region of veins, especially heavy along costal margin. An oblique black streak in apex of costal plaque. Apical cells more or less clouded with fulvous.

Inner Male Genitalia. (Pl. VIII.)

Distribution. Kansas.

Erythroneura vitis Harris is another of the species which commonly infests the grape. It is easily distinguished by its two broad scarlet bands and dusky apical cells. Five varieties have been described.

KEY TO VARIETIES OF *VITIS*.

PAGE

A. Anterior crossband extending beyond apex of scutellum, at least along costal margin; middle crossband usually broader, extending to or beyond apex of clavus.
  B. Middle crossband bounded by nearly or quite interrupted pale bands.
    C. Ground color between crossbands white or creamy-white; crossbands red to dusky. *vitis*.. 144
    CC. Ground color between crossbands deep canary-yellow; crossbands tan to fulvous. var. *flava*.. 144
  BB. Pale areas bounding middle crossband interrupted or replaced by red markings.
    C. Crossband bounded by a wreath of pale spots, about three on each tegmen anteriorly and four posteriorly. var. *corona*.. 145
    CC. Crossband bounded anteriorly by a more or less interrupted pale area, sometimes reduced to a single spot on clavus, and posteriorly broadly joined to red markings on crossveins, leaving a pale area only at and adjacent to apex of clavus; the tegmen therefore chiefly red, sometimes with only two pale areas at base and apex of clavus, which latter the middle crossband usually does not reach. var. *bistrata*.. 145
AA. Anterior crossband not extending beyond apex of scutellum, middle crossband narrower, not reaching apex of clavus. var. *stricta*.. 145

Erythroneura vitis Harris.

Tettigonia vitis Harris. Encyclopedia Americana, VIII, p. 43; 1831.
Typhlocyba comes var. *vitis* Gillette. Proc. U. S. Nat. Mus., vol. XX, p. 761; 1898.

Color Pattern. The following description is by McAtee:[9] "Vertex yellowish with two parallel orange reddish vittæ, broader and deeper colored behind, sometimes almost wholly red; pronotum scarlet with a short median vitta in front, extreme anterior margin, and obsolete discal parentheses, pale yellow; scutellum chiefly scarlet, basal triangles and basal median vitta sometimes pale; tegmen with three crossbands, the anterior scarlet extending well beyond apex of scutellum, the median scarlet (often paler red to dusky within), broad, from middle to near tip of clavus; those on the two tegmina together forming a large roundish saddle spot, sometimes slightly projected along the sectors or connected to anterior band or posterior ramose line at a few points, and terminating laterally at costal plaque, which is blackish posteriorly and sometimes followed by a yellowish area; third crossband made by the blackish apical cells which are hyaline basally; a ramose red line on cross veins and adjoining parts of longitudinal veins."

Inner Male Genitalia. (Pl. VIII.)

Host Plants. Grape.

Distribution. Ontario, Quebec, Maine, New York, Connecticut, Pennsylvania, Maryland, Virginia, Tennessee, North Carolina, South Carolina, Mississippi Ohio, Michigan, Indiana, Wisconsin, Illinois, Missouri, Iowa, Nebraska, Kansas, Colorado.

Erythroneura vitis var. *flava* Robinson.

Erythroneura vitis var. *flava* Robinson. Can. Ent., vol. LVI, No. 3, p. 62; 1924.

Color Pattern. Vertex, pronotum and scutellum buff, a brownish area on disk of pronotum. Tegmina, ground color canary yellow, light brown band across base, a broader reddish-brown band across middle. Cross veins faintly red; apical cells smoky.

Distribution. Minnesota.

9. Trans. Am. Ent. Soc., XLVI; 1920.

Erythroneura vitis var. *corona* McAtee.

Erythroneura vitis var. *corona* McAtee. Trans. Am. Ent. Soc., LVI, p. 304; 1920.

Color Pattern. "Similar to variety *vitis*, but middle crossband on tegmina connected by red lines along longitudinal veins to anterior crossband and to ramose marking on cross veins, so that the saddle mark appears to be surrounded by a wreath of pale spots." (McAtee.)

Distribution. Maryland, Illinois, Kansas.

Erythroneura vitis var. *bistrata* McAtee.

Erythroneura vitis var. *bistrata* McAtee. Trans. Am. Ent. Soc., XLVI, p. 305; 1920.

Color Pattern. "This variety illustrates the extreme degree of erythrization of this species I have seen; the vertex may be pale yellow, but usually it has the normal pair of vittæ strongly developed, and it may be almost entirely red; the pronotum is red, with touches of pale yellow on anterior border, with a dot in middle, and traces of pale yellow at about two points on the hind margin; scutellum red, with a pale median vitta enlarged at apex; tegmen often entirely red, with the exception of a pale spot at inner base of clavus and another involving apex of clavus and adjoining corium; there may be other traces, however, of both anterior and posterior pale bands, and the costal plaque, except for its posterior blackish marking and an equal sized area behind it and the bases of the apical cells, are pale; under surface and legs chiefly pale yellow. (W. L. McAtee.)

Distribution. Maryland, Illinois.

Erythroneura vitis var. *stricta* McAtee.

Erythroneura vitis var. *stricta* McAtee. Trans. Am. Ent. Soc., XLVI, p. 305; 1920.

Color Pattern. "Ground color whitish-hyaline to pale yellow; vertex chiefly pale, sometimes with a few touches of red, pronotum and scutellum chiefly red to dusky, the latter sometimes pale medianly and at tip; tegmen with three crossbands, the anterior red, narrow, not exceeding scutellum, the middle one narrower than in the other varieties, usually nearly quadrilateral in shape; the posterior much as in last variety, pale spaces between the bands broader than in the other varieties; usually half or more of costal plaque bluish or blackish, often overlaid by opaque-white; lower surface stramineous to pale yellow." (McAtee.)

Distribution. Pennsylvania, Maryland, Indiana, Iowa, Kansas, Colorado.

McAtee describes also a yellow form of this variety, which I am including here:

"Vertex, pronotum and scutellum chiefly yellow, the last two more or less overlaid by brownish; tegmen with three crossbands, the anterior yellow to red, involving base of corium not beyond apex of scutellum and narrowly extended along costa, the median broad, semielliptical, red, duller within, terminating laterally in a blackish marking on posterior third of costal plaque; posterior crossband formed by dusky clouds in apical cells; a branching red line on cross veins and adjacent parts of longitudinal veins. Legs and face pale yellow; mesosternum black; body slaty with lighter edgings, varying to wholly pale yellowish."

Erythroneura lacta Robinson.

Erythroneura lacta Robinson. Can. Ent., vol. LVI, No. 3, p. 62; 1924.

Color Pattern. Vertex, pronotum and scutellum creamy-white. Tegmina opaque-white, relieved by the following color pattern: Vertex, two very pale yellow spots on posterior margin; pronotum, an indistinct yellow Y-shaped vitta; scutellum, a light yellow spot in each basal angle and at tip. Tegmina, on clavus an orange spot at base, a red triangular area at middle, and an orange spot at tip; on corium an orange spot at base, costal plaque bordered on front and inner margins by a narrow orange mark, at the middle of which is a short red extension to meet the claval suture and at its caudal end a faint orange vitta which proceeds in a widening line to base of cell M_4. Cross veins more or less reddish; a black spot in the apex of cell R_3 and base of cell M_4.

Inner Male Genitalia. (Pl. VII.)

Host Plants. Grape.

Distribution. Ontario, Kansas.

Erythroneura omaska Robinson.

Erythroneura omaska Robinson. Can. Ent., vol. LVI, No. 3, p. 62; 1924.

Color Pattern. Vertex orange, with a median vitta and four lateral spots of yellow. Pronotum white, a Y-shaped median vitta with yellow arms and orange base, a diamond-shaped orange vitta behind each eye. Scutellum creamy, with yellow spots outlined in red in each basal angle and red tip. Tegmina, ground color opaque white, with the following orange markings: On clavus, a vitta in touch with basal half of claval suture, constricted in the middle and barbed at caudal end and a spot at tip; on corium, an oblique streak near the base, a line bordering front and inner margins of costal plaque, in the middle of which is an abrupt extension to the claval suture, then a line proceeding in a widening paler vitta to the base of cell M_4. Cross veins vermillion; apical cells, white bordering cross veins and remainder smoky. An oblique dark streak in the apex of costal plaque, and a black spot in the apex of cell R_3 and base of cell M_4.

Inner Male Genitalia. The apical process of the œdagus is long and heavy and strongly bifurcate. The apices of the styles are also very characteristic of this species. (Pl. VII.)

Distribution. Kansas.

Erythroneura acuticephala Robinson.

Erythroneura acuticephala Robinson. Can. Ent., vol. LVI, No. 3, p. 61; 1924.

Color Pattern. General ground color yellowish-white with light red markings as follows: Vertex, a long, very slender inverted U-shaped vitta; pronotum, a slender Y-shaped vitta with short arms and long heavy base, a long vitta on each lateral margin; scutellum, a large spot entirely red or outlined in red in basal angles, a spot at tip. Tegmina, on clavus a vitta beginning half way between humeral and inner basal angles and proceeding half the length of the claval suture, where it becomes enlarged and projects inward and touches the tegminal suture; on corium an oblique vitta beginning about half way between humeral angle and costal plaque and proceeding inward to a

point one-third along the claval vitta, an oblique vitta beginning as a narrow streak in front of the costal plaque, soon widening and proceeding back to the base of cell M_4, and connected with the tip of the claval vitta by a short band. Cross veins red; apical cells smoky; an oblique dark mark in the apex of costal plaque; a black dot in the apex of R_3 and the base of M_4. The marks on the tegmina tend to be lighter colored with darker margins.

Inner Male Genitalia. (Plate VIII.)

Distribution. Kansas.

This is a slender species with very pointed vertex.

Erythroneura scripta Robinson.

Erythroneura scripta Robinson. Can. Ent., vol. LVI, No. 12, p. 290; 1924.

Color Pattern. General ground color of dorsum milky-white, except vertex which is ivory-white, and marked with red as follows: Vertex, a large O-shaped median mark to which are joined two smaller lateral ones. Pronotum, a T-shaped vitta with two short lateral spots behind the eyes. Scutellum, a spot in each angle. Tegmina, on clavus a small spot near base, a triangular area at middle with a black spot in the inner angle; on corium, two spots at base which are sometimes fused, an interrupted line around the costal plaque, a spot between the latter and the claval suture, an oblique line running from the black streak at apex of costal plaque to the base of cell M_4. Radius and media in region of cross veins red. Apical cells smoky except a clear outline along the base. A black spot in apex of cell R_3 and base of cell M_4.

Inner Male Genitalia. (Pl. V.)

Distribution. Kansas.

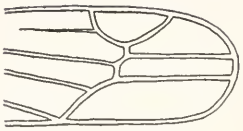
Erythroneura fraxa Robinson.

Erythroneura fraxa Robinson. Can. Ent., vol. LVI, No. 12, p. 292; 1924.

Color Pattern. Ground color creamy-white, with the following pattern in red: Vertex, a very broad median vitta, which may consist of two narrow vittæ fused together. Pronotum, a narrower median vitta bifurcate at anterior end, a broader lateral vitta behind each eye. Scutellum, a large triangular area in each basal angle and a small dot at tip. Tegmina, an unusually broad area at the humerus, probably made up of two vittæ fused together; from this a line extends inwardly to the tegminal suture midway along the clavus. Another line arises before the costal plaque as a very fine streak, soon widens greatly and proceeds along the inner margin of the plaque and then inwardly to the base of cell M_4. These two lines, the claval and corial, are connected in the region of the costal plaque by a short band and make one continuous vitta from the humerus to the crimson cross veins. Apical cells dusky; a black spot in the apex of cell R_3 and base of cell M_4.

Distribution. Kansas.

PLATE I.



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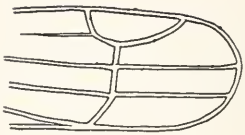

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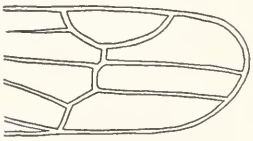
Maculata Group



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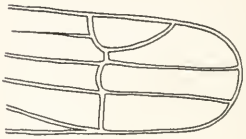
Vulnerata-Scutelleris Group



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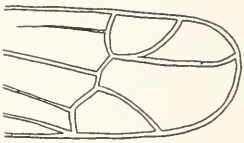
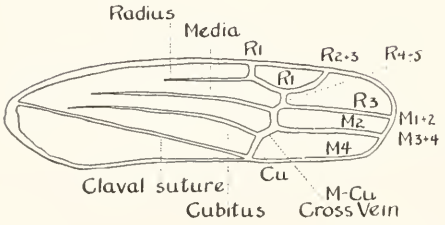
Comes Group



Genus Typhlocyba



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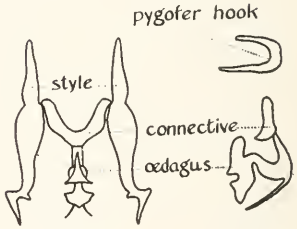

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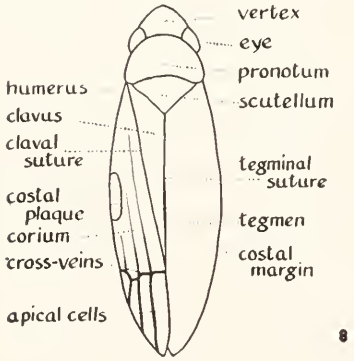

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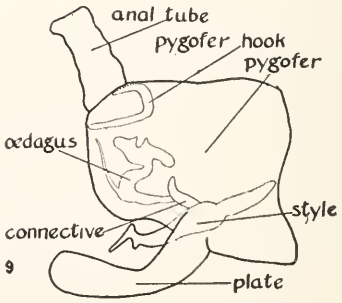

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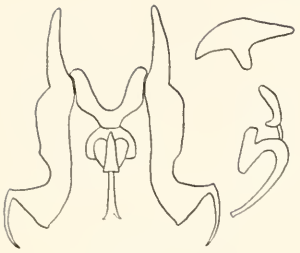
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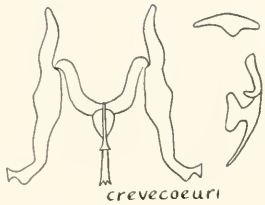
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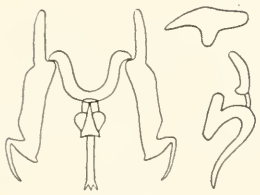
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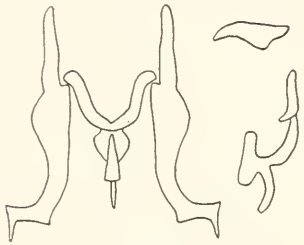
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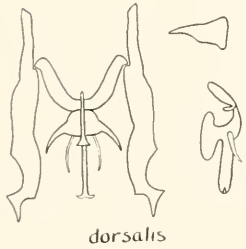
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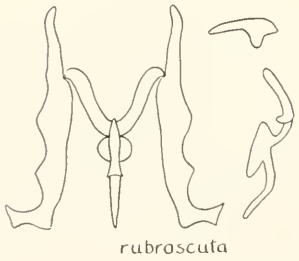
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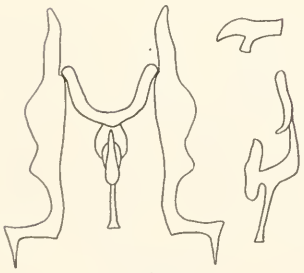
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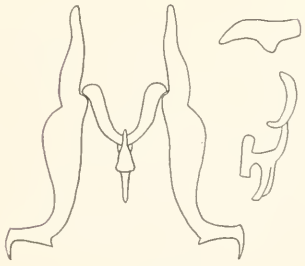
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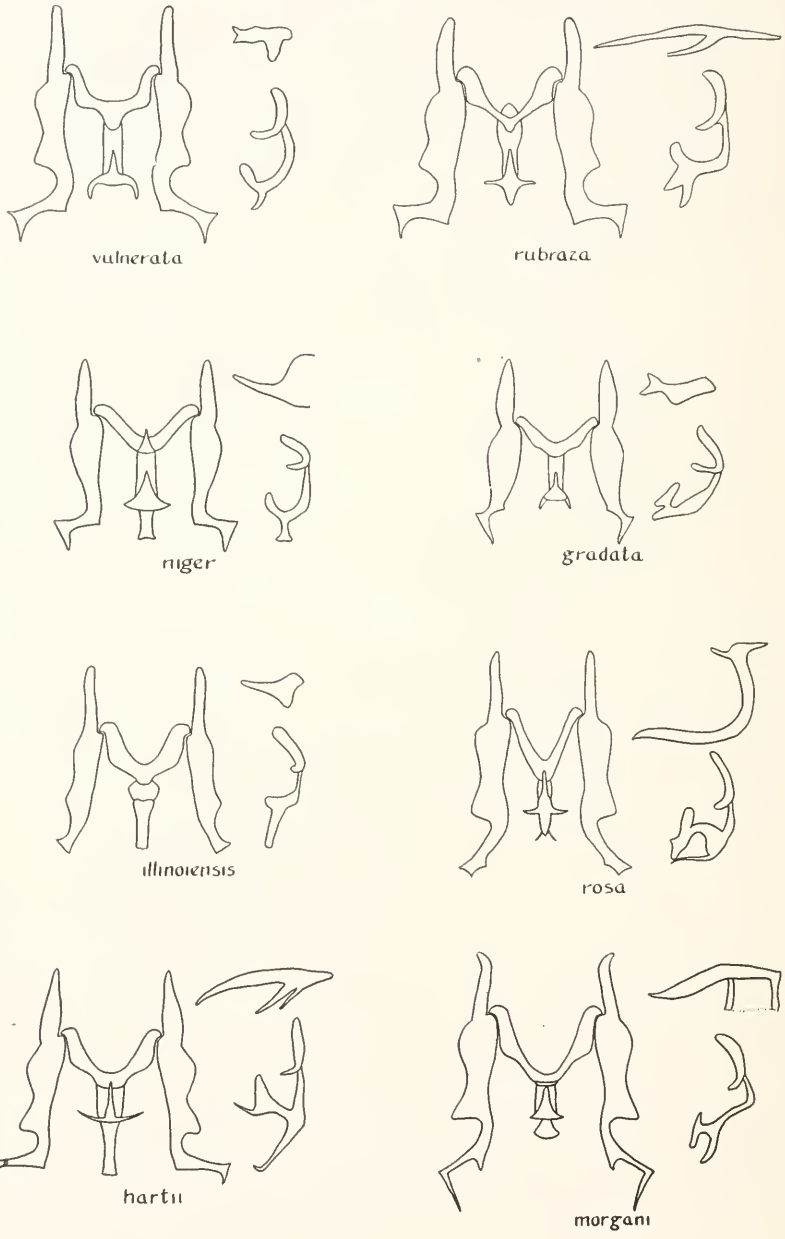
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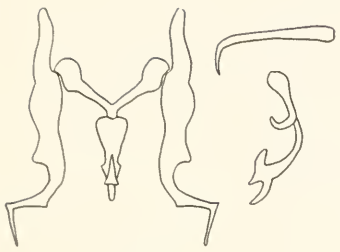

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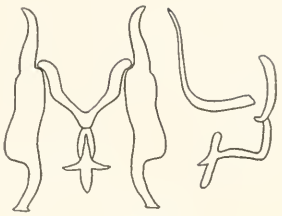

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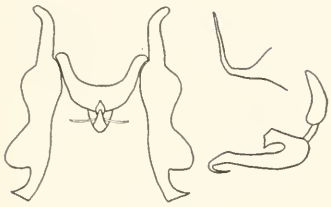

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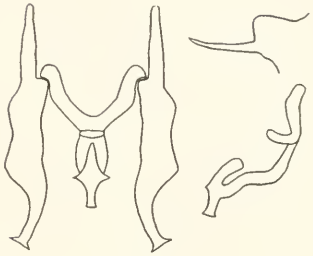

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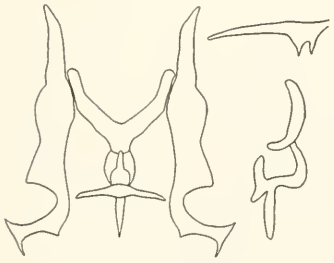

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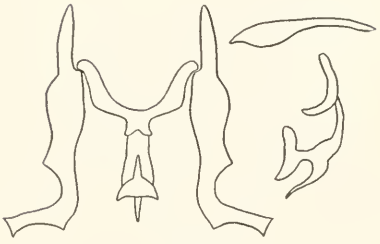

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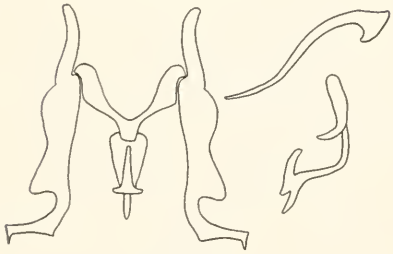

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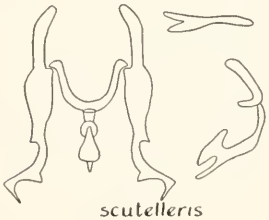

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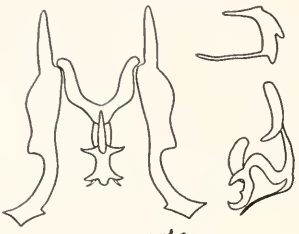
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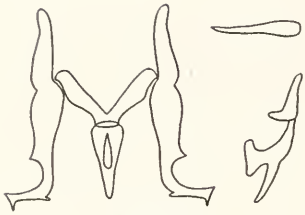
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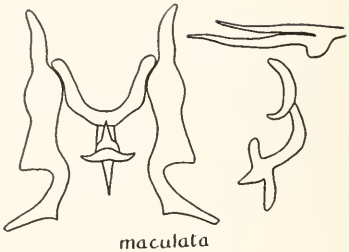
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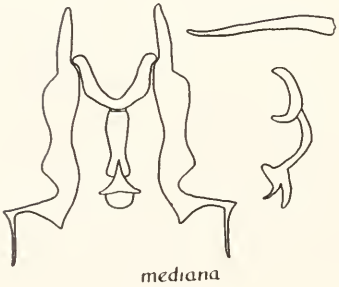
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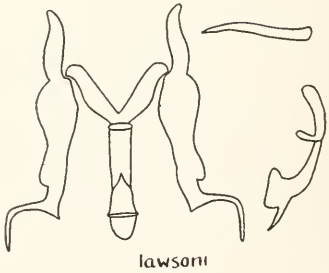
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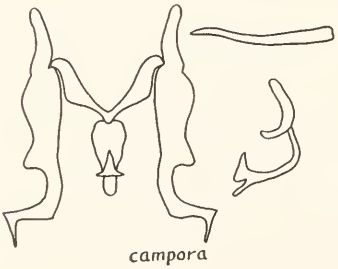
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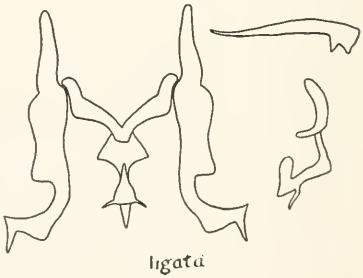
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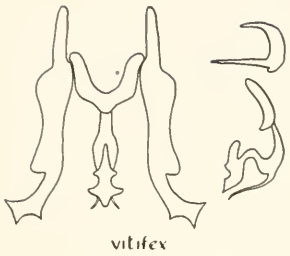
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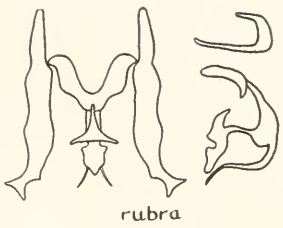
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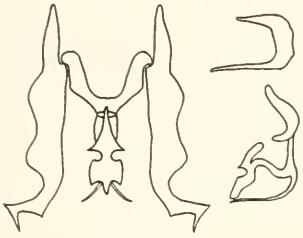
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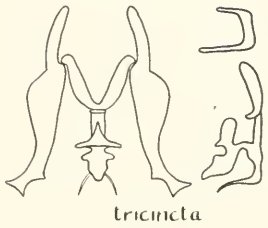
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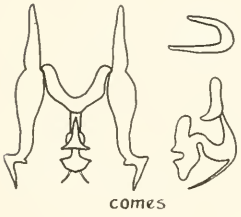
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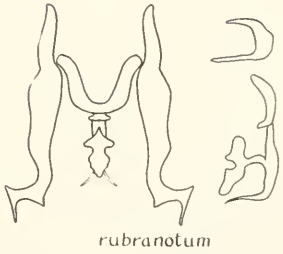
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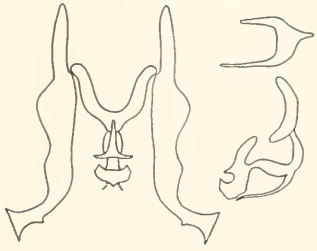
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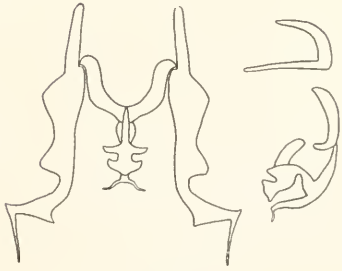
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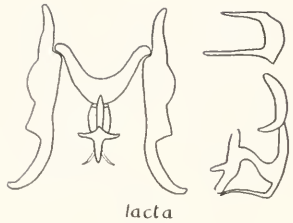
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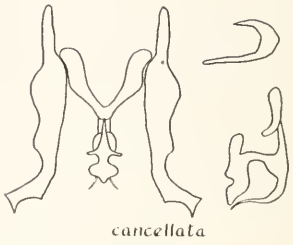
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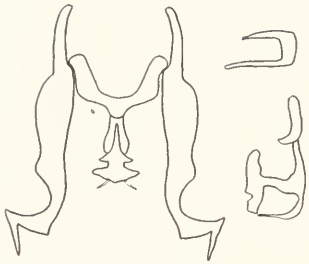
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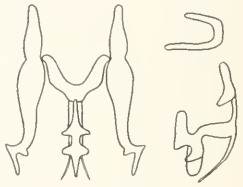
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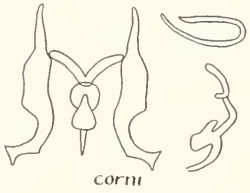
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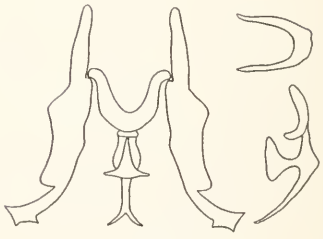
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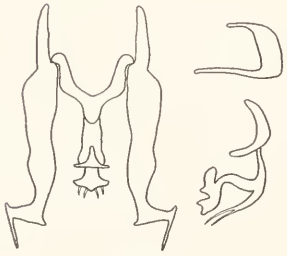
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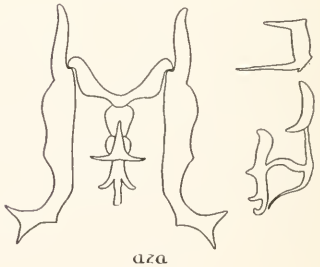
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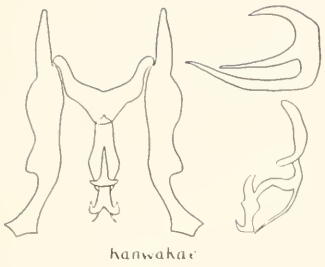
PLATE VIII.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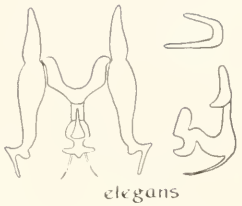
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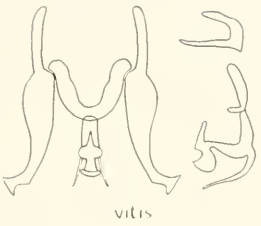
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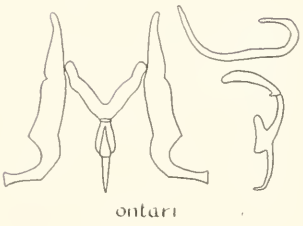
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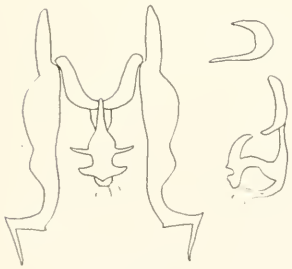
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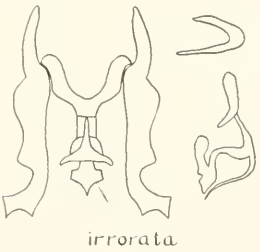
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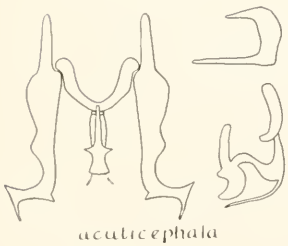
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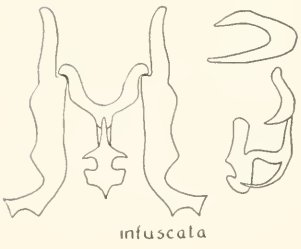
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Studies on the Eggs of Some Reduviidæ (Heteroptera).

P. A. READIO, Department of Entomology.

DURING the past few summers the writer has been working on the biology of various Heteroptera, and has more than once been struck with the unusual appearance of the eggs of the Reduviidæ. Not only are the individual eggs of some of the species very much decorated, but the ornamentation is different in the egg of each species, and is in fact characteristic for the species. This paper presents a general discussion of the eggs in this family, followed by descriptions and notes on the eggs of each species studied.

The family Reduviidæ is one to which no uniform habitat can be assigned, and we find the eggs in as wide a variety of situations as we find the insects themselves. Thus some tree-inhabiting species attach their eggs to the trunks and larger branches of trees. Others show a preference for the twigs, and still others attach their eggs to the leaves themselves. Those species which live on the lower foliage of open, sunny fields usually attach their eggs to the stems and leaves of grasses and weeds. Several ground-inhabiting species lay their eggs partly buried in the soil underneath protected rocks, and still others attach their eggs to the rocks themselves, usually on the lower side. Thus, the only generalization possible is that the eggs are usually found in the same situations in which we find the adults, however varied these may be.

The time of year when the eggs are laid also varies a good deal for the different species in the family. At least one species we know of, *Arilus cristatus* (Linnæus), lays its eggs in the fall and spends the winter in the egg stage. A good many reduviids spend the winter as adults and lay their eggs in spring and early summer, and a third group spend the winter as nymphs, become adults in the spring and summer, and lay their eggs then.

In the grouping and attachment of the eggs we find no more uniformity than before. *Reduvius personatus* (Linnæus) deposits its eggs singly in out-of-the-way places, with no sign of definite arrangement or attachment. *Melanolestes picipes* (Herrich-Schäffer) also lays its eggs singly, but in this case the bases are inserted into the ground for support. *Pselliopus cinctus* (Fabricius) is another species which lays its eggs one at a time, but each egg is attached to its supporting surface by a cement. Other species lay their eggs in groups of various numbers, from three to fourteen in the case of *Sinea spinipes* (Herrich-Shäffer), up to over a hundred and fifty in the case of the *Arilus cristatus* (Linnæus). The grouping of the eggs in masses, and the amount of cement used to attach and protect the eggs also varies. This lack of uniformity does not permit of generalizations, but demands separate descriptions for each species.

The more general features of the structure of the egg are uniform throughout the family. The egg is usually cylindrical or elongate-ovate, and in every species in which the egg has been studied there is, at one end, a definite cap which is pushed off in the hatching process. This cap is plain, or it may be ornamented, as is more often the case, in a variety of ways. The rim of the egg, surrounding the cap, may also be ornamented by an extension of the chorion. It is the ornamentation of the cap, and the extension of the chorion around the cap, that give the eggs in this family their unique appearance.

The eggs of twenty species and one variety of our United States fauna are discussed. These are distributed over six subfamilies and fourteen genera. While this is only a very small representation of the total number of species found in the United States, yet a surprising variety in form is found to exist. An attempt was made to determine whether members of the same subfamily, and of the same genus, show the similarity that one would expect, and while the number of species available was not as large as could be desired for this study, yet the indications are that there is subfamily and generic resemblance in the eggs.

Subfamily PLOIARIINÆ.

The eggs of three species of this subfamily have been studied at first hand, and a description of those of a fourth species is found in the literature.

Empicoris rubromaculatus (Blackburn).

I have received recently from Mr. McAtee, of the United States National Museum, some specimens of the eggs of this species. Unfortunately they are not in as good condition as one would wish, being somewhat shrunken and distorted, and consequently do not lend themselves well to exact description. Certain things concerning them may be mentioned, however. The eggs are about six-tenths of a millimeter in length, and about one-half as wide. The color is dark brown; the shape cylindrical. At one end there is a definite, circular cap. In their present condition they show no evidences of ornamentation, although inasmuch as the eggs of the other members of this subfamily are ornamented, it seems reasonable to question, at least, whether or not these have lost their ornamentation.

Empicoris errabundus (Say).

The writer has not been able to obtain the eggs of this species, but a description of them prepared by Provancher[13] is available. A free translation of this description is given for the sake of making this work as complete as possible:

"This insect, taken at Trois-Rivieres and transported alive in a small box, deposited its eggs during the journey. The eggs, ten in number, were kidney-shaped, hollowed out on their inner side and bearing four or five white lines on the back. One of the extremities is terminated by a little cap preceded by a slight constriction, and bearing at the end a projection in the shape of a coronet. They are attached to the pedicel, not by an extremity, but by the dorsal surface; that is, the pedicel is prolonged on the pasteboard and adheres there in the form of a very thin line, bearing each egg attached on its middle by its dorsal surface. From the exterior appearance they might easily be mistaken for the seeds of umbelliferous plants or the anthers of certain flowers. It was only after having submitted them to the microscope that we were able to convince ourselves that they were animal, and not plant productions."

From this description it can be seen, at least, that the eggs of this species are like those of the two following species, for which figures are given, in that they are ornamented both on the body and on the cap, and are attached along one of the long surfaces of the egg rather than at the base.

Emesaya brevipennis (Say).

(Pl. X, fig. 10.)

The eggs of this species have been studied, figured and described by several observers. The writer has abundant material of the eggs of this species collected in Kansas. The following description of the eggs is given by McAtee and Malloch:[11]

"The eggs of this species are about 2 millimeters in length, long-elliptical in outline, the opercle with a large central truncately conical tubercle, the periphery of which is more or less eroded at the base; the main body of the egg is black in ground color, somewhat compressed and with longitudinal rows of membranous, saw-tooth-shaped exfoliations, the bases of which are almost continuous; these lines of projections are arranged more or less in concentric ellipses on the flat sides of the egg."

These eggs are laid singly, attached to the bark of trees, particularly evergreen (Weed),[7] or to rafters (Howes).[8] The writer has collected eggs which were attached to the rafters of a shed, and has also found them attached to a spider's thread. Each egg is attached along its long surface, near the base. The egg is inclined and forms an angle with the surface upon which it rests.

Metapterus fraternus (Say).

(Pl. X, fig. 11.)

The eggs of this species have been figured by McAtee and Malloch,[11] but no description accompanies their figure. The following description has been prepared by the writer: Length, 1.4 mm.; width of top, .3 mm. Shape, rather elongate, cylindrical, with axis of cylinder slightly curved; base rounded, apex truncate, surmounted by a cap with central conelike projection; longitudinal, flangelike ridges on body of egg for whole length, ridges united in pairs at base of egg, extended a little beyond apex of body of egg; cap with peripheral, reticulated area, and central conelike structure, this formed of projections from reticulated area. Color of body of egg dark brown and shining, of flangelike longitudinal ridges whitish, of reticulated area and cone of cap greyish-white.

These eggs, as in the preceding species, are laid singly and attached in the same manner. In the laboratory they were attached to bits of roots, and to the sides of the rearing dishes.

The similarity between the eggs of the last two species mentioned is quite evident. They are both comparatively elongate and slender, both provided with longitudinal ridges, and both fitted with a conical cap. Eggs of the members of no other subfamily resemble them except in very general features.

SUBFAMILY STENOPODINÆ.

The eggs of only one species of this subfamily are available for study. No previous reference to the eggs of this species, nor to the eggs of any other United States species of this subfamily have been found.

Oncocephalus apiculatus Reuter.

(Pl. X, fig. 4.)

The eggs described here were obtained this past summer from a wingless female which answers to Reuter's[14] description of the female of this species.

Description of egg: Length, 1.4 mm.; greatest width, 1 mm.; diameter at cap, .7 mm. Color, uniform dull white, surface reticulated. Body of egg nearly spherical in shape, slightly longer than wide; base smoothly and evenly rounded, apex truncate and with a distinct cap; upper extremity narrowed to a distinct collar, irregular fringe of extension of chorion above collar; cap within fringe, circular in outline, somewhat concave; periphery of cap with a regular and closely spaced circle of erect appendages of uniform length, produced rimlike beyond irregular fringe of extended chorion; convex disk of cap with clothing of rather closely spaced hair-like appendages.

This egg is quite different from that of any other species studied. Whether it is typical for the subfamily or not will be seen when eggs of other species of the subfamily are discovered.

The eggs of this species are inserted into the ground, with only the upper end visible, as is the case in the subfamily Piratinæ.

SUBFAMILY REDUVIINÆ.

The eggs of only one species of this subfamily, *Reduvius personatus* (Linnæus), have been obtained for study. However, figures and descriptions of two other species from the United States are available for comparison. These are *Triatoma sanguisuga* (Le Contte) and *Triatoma protracta* (Uhler). The eggs of these three species are similar and differ from the eggs in other subfamilies in lacking the usual ornamentation of the top of the egg and of the cap. In fact, they are the simplest of the reduviid eggs studied.

Reduvius personatus (Linnæus).

(Pl. IX, fig. 1.)

This is the "Masked bedbug hunter," a European species now well established in this country, and well known as an occasionally troublesome inhabitant of houses.

The following description of the egg is taken from E. A. Butler,[3] who in turn quotes from Leuckhart:

"The eggs of *Reduvius personatus* recall by their form those of the Pentatamidæ. They are oval and obese, fully 1 mm. long, narrowed toward the base and furnished at the flatter end with a rather flat lid which carries a groove. The brownish yellow chorion is decidedly hard. The outer surface is quite smooth and shining, the inner, on the contrary, is of finely granulated appearance. No further structure can be seen except here and there a minute canal leading to the under surface of the chorion, and undoubtedly intended to bring air into close contact with the yolk and embryo. The outer margin of the groove round the lid is lengthened into a thin, projecting, rimlike lamella, on the inner wall of which there are a number of vertical prominences. These are the micropyles, and their number may amount to about eighty. In each of these micropyles can be distinguished two parts, one outer and cupshaped, the other inner and canal-shaped. The former is attached all along to the inner surface of the lamella; it begins with a transverse opening close beneath the upper margin of the rim, and gradually narrows till it reaches the bottom of the groove. The free, cupshaped part is continued into a canal which penetrates the chorion. Out of the lower end of the cup there arises a fine passage, the above-mentioned under segment of the micropyle, which runs down for a short space in the substance of the chorion, and then after a short bend opens on to the inner surface."

I obtained the eggs of this species by isolating adult females which were found here and there in houses. Evidently these females had mated previously, for in every case the eggs obtained were fertile.

Triatoma sanguisuga (Le Conte).

(Pl. IX, fig. 2.)

This species, because of its tendency to feed on human blood, is known as the "Blood-sucking cone-nose," or "Big bedbug." Heidemann,[5] in his work on the eggs of the Heteroptera, gives the following descriptive notes concerning the eggs of this species:

"Egg, 1 mm. long; ovate, chorion somewhat flattened near the lower end; the inner side of the extension of the rim shows the chorial processes plainly; outer surface very fine granulate, pearly white."

Miss Kimball,[9] in her paper on the life history and habits of this species, gives the following description of the egg:

"The egg, which is about the size of a mustard seed, and of a yellow color, is peculiar in its shape, resembling that of a bottle with a thickened rim

around the top, giving this portion the appearance of a stopper, especially after the egg hatches, as the insect pushes out this tiny, saucerlike top, which falls to the ground. Though yellowish when first laid, the egg soon changes in color to pink, and then to red, as the insect develops within, until just before hatching, when the segments of the body can be seen through the transparent shell."

Triatoma protracta (Uhler).

I find a brief note concerning the eggs of this species given by Herms.[6] This is quoted:

"*Triatoma protracta* (Uhl.) deposits large whitish eggs in small numbers (three to six) at a laying under laboratory conditions in almost any sort of vial or container in which the female may happen to be imprisoned. The eggs are deposited during midsummer and late summer at least until early September in Berkeley (California) indoors."

Herms' photograph of the egg of this species shows that in essential structure it is similar to that of the preceding species.

The significant thing in the structure of the eggs of the species of *Triatoma* is that the cap itself is not ornamented, and that the chorion in the region surrounding the cap is not ornamented. In this respect the eggs resemble those of *Reduvius personatus* (Linnæus), a member of the same subfamily. However, the eggs in this subfamily are the only ones studied where the structure is so simple

SUBFAMILY PIRATINÆ.

The eggs of two species of this subfamily have been obtained for study, and it is interesting to note that they are similar both in structure and in place and manner of deposition, while at the same time they differ in these respects from the eggs in the other subfamilies studied. The two species in which the eggs have been studied are *Melanolestes picipes* (Herrich-Schäffer) and *Rasahus biguttatus* (Say). No previous references to the eggs of these species nor to the eggs of any other United States species of this subfamily have been found.

Melanolestes picipes (Herrich-Schäffer).

(Pl. IX, figs. 3 and 4.)

This is a very common, black reduviid taken commonly on the ground under rocks and logs, and occasionally coming to lights at night. Its ability to inflict a poisonous bite, and its willingness to do this in defense, has given it, along with several other reduviids of similar habits, the name, "Kissing bug."

Description of egg: Length to extension of chorion, 2.5 mm.;

greatest width, 1.3 mm. to 1.5 mm.; diameter of extension of chorion, 2.2 mm. to 2.5 mm. Color, entirely dull white at first, later darker with progression of incubation, appendages and segmentation visible. Shape, elongate oval; distinct collar at upper end, beyond this chorion extended in a series of flat, elongate scales, these arranged radially around cap, each scale narrowed slightly towards tip, tip rounded; scales toothed along margins, forty to fifty radiating scales present. Cap more or less sunken in middle, radiating scales at periphery, these produced for a short distance over the scales of the extension of the chorion, closely pressed to these and attached, one scale of cap to each scale of extension of chorion.

The eggs of this species were obtained by collecting adults in their natural habitat under rocks early in the spring, and confining them in suitable cages with insects supplied as food. Eggs were also collected in the field. Both in the laboratory and in the field the eggs were placed in the ground, the entire lower portion of the egg being buried, with only the white, starlike top visible. They are always laid under some protecting rock or log.

Eggs were also obtained from the variety *abdominalis* (Herrich-Schäffer) of this species. These eggs were similar to those of the typical individuals of the species.

Rasahus biguttatus (Say).

(Pl. IX, figs. 5 and 6.)

This insect, known commonly as the "Two-spotted corsair," is abundant enough to have caused some comment in economic literature because of its bite, which, the writer can attest, is very painful.

Description of egg: Length to extension of chorion, 2.5 mm.; greatest width, 1.3 mm.; length of extension of chorion, 0.6 mm. Color, dead white at first, darker later, finally with red eyes, dark legs, and red and white striped abdomen of unhatched insect visible. Shape, elongate-ovate, ornamented in the region of cap; collar at top of egg, beyond collar chorion extended in a series of curved scales, about forty in number, scales with no teeth along margins; central area of cap with a very distinct, erect spine, periphery of cap with long, slender scales; scales of cap produced over scales of extension of chorion, attached to them, equal in number to the scales of the extension of the chorion.

This egg is quite similar to that of the closely related *Melanolestes picipes* (Herrich-Schäffer). The two eggs differ: (1) in that the scales forming the extension of the chorion are longer, more nearly horizontal, and bear small teeth along their lateral margins in

Melanolestes picipes (Herrich-Schäffer), whereas they are shorter, more nearly erect, and bear no teeth in *Rasahus biguttatus* (Say); (2) in that the radiating scales of the cap are broader and shorter, not extending over more than one-third the length of the chorion scales in *Melanolestes picipes* (Herrich-Schäffer), and are longer and narrower, extending almost or quite to the tip in *Rasahus biguttatus* (Say); and (3) in the presence of a central spine in *Rasahus biguttatus* (Say), which is lacking in *Melanolestes picipes* (Herrich-Schäffer).

The eggs of this species are placed in the ground under the protecting rocks which shield the adults. Sometimes the eggs are entirely buried, sometimes standing erect but buried with the exception of the extending scales of the chorion, and sometimes lying flat, partially buried.

SUBFAMILY APIOMERINÆ.

The eggs of only one species of this subfamily, *Apiomerus spissipes* (Say), are represented in my collection, though a description and figure of the egg of another species, *Apiomerus crassipes* (Fabricius), are available for comparison. The eggs of these two species of the same genus are quite similar, and at the same time different from the eggs in the other subfamilies studied.

Apiomerus spissipes (Say).

(Pl. X, figs. 9 and 12.)

This is a broad, stout, hairy insect, found as an adult on the stems and leaves of tall weeds and other plants, and as a nymph, at least by the writer, under rocks. Morgan [12] studied the life history of this insect as a possibly important natural enemy of the cotton boll weevil, but concluded that the insect was economically insignificant in this respect.

Morgan [12] gives the following description of the egg:

"The egg is cylindrical, finely punctured, and varies in color from a bright yellow when first deposited to a light brown just before hatching. The collar is shining white. The dimensions of the egg are as follows: Width at narrowest part—just below collar—0.5 mm.; greatest width, 0.64 mm.; length below collar, 1.51 mm.; length of collar, 0.26 mm.; total length, 1.77 mm. The width of the collar varies from slightly less than the greatest width of the egg to slightly more."

I wish to add to this description by saying that the collar referred to above is formed as the extension of the chorion by longitudinal scales connecting with each other so as to form the membranelike

collar. The cap of the egg is sunken in the middle, somewhat elevated around the outer rim, and is fringed with conspicuous white scales.

Morgan gives the following additional notes concerning the eggs:

"The eggs are laid in masses, though as few as 10 were laid in one instance and 23 in another. Individual records are as follows: 37, 41, 49, 54, and 64. The usual place for oviposition is the underside of a leaf, near the top of the plant. Egg masses have been collected in this position from *Ambrosia* sp. and *Helianthus* sp. The eggs are placed so close to each other, side by side, that in the center of the mass, instead of being cylindrical, they are usually more or less hexagonal."

It is worth mentioning that the writer was unable to find the eggs of this species on the leaves of plants in Kansas, but did find several egg masses attached to rocks on the ground. In one case an egg mass was attached to the upper surface of a rock which was covered by a superimposed rock, and in another case the egg mass was found on the under side of a rock which rested on the surface of the ground.

Apiomerus crassipes (Fabricius).

As mentioned above, the writer has not been able to find the eggs of this species, but has found a figure and descriptions of them which are valuable. The species is very closely related to the preceding. Heidemann[5] gives the following description of the eggs of this species:

"Egg, 1.8 mm., oval-elongate; color, dark brown; the extension of the chorion at upper egg-pole composed of longitudinal fine scales connecting with each other, yellowish around the rim and white at the edge; the cap rather low, crowned with white scales, of which those on the inside circumference are brown."

Uhler,[15] in speaking of this species, says that it lays its eggs on the twigs and bark of the common pine trees, and that these hatch during the early summer.

It is impossible for the writer, having at hand only descriptions and figures of the eggs of this species, to pick out any essential difference between it and the preceding species. It is probable that if the two eggs were compared side by side some difference would be apparent. It should be remembered, however, that the two species are very closely related indeed, difficult of separation even as adults.

It is regrettable that there is no more material of this subfamily available, but with the limited material at hand, it is evident that the eggs of this subfamily are different from those in other subfamilies, and represent, in fact, a distinct type.

Subfamily HARPACTORINÆ.

The eggs of eight species of this subfamily, distributed among five genera, have been studied. These species are *Zelus exsanguis* (Stal) and *Z. socius* (Uhler), *Pselliopus cinctus* (Fabricius) and *P. barberi* Davis, *Arilus cristatus* (Linnæus), *Acholla multispinosa* (DeGeer), *Sinea diadema* (Fabricius), and *S. spinipes* (Herrich-Schäffer). In every case where more than one species of a genus has been studied, generic resemblance is apparent. From a study of the figures presented it is at once apparent that there is no great degree of uniformity in the eggs of the subfamily as a whole. While it is true that the eggs *Pselliopus* and *Zelus* resemble each other, as do also the eggs of *Arilus*, *Acholla* and *Sinea*, yet the eggs of these two groups do not resemble each other any more closely than they do those of the Apiomerinæ. To be sure, this is a large and somewhat heterogeneous subfamily, which fact probably accounts for the lack of uniformity in the eggs.

Zelus exsanguis (Stal).

(Pl. X, figs. 5, 6 and 7.)

From the number of references to this insect, either under this name, or its synonym, *Diplodus luridus*, it must be very common. Indeed, the writer has found it to be very common in woods in Kansas. It is a very slender, long-bodied, green species, with very long, slender legs. The eggs have been described by Kirkland,[10] but this description is not available. Heidemann[5] gives the following brief notes:

> "The sticky secretion the insect uses for protecting its eggs covers sometimes also the cap, leaving only in the center a small opening; chorial processes close together, club-shaped."

The writer wishes to add to this description by giving the dimensions of the egg: Length, 2 mm.; greatest width, 0.7 mm.; length of extension of chorion, 0.3 mm.

The eggs of this species were found very commonly in the field, and also were easily obtained in the laboratory by isolating adult females. The eggs are laid in very compact, five- or six-sided masses, each egg standing on end, and pressed so tightly by the surrounding eggs as to have a definite hexagonal shape. The eggs are embedded in, and, in fact, almost entirely surrounded by a cement, this species being the most generous in this respect of all those studied. The egg mass is brown when viewed from the side and white when viewed from the top. Each egg mass contains in the neighborhood of fifty eggs.

Zelus socius (Uhler).

(Pl. X, fig. 8.)

The eggs of this species, a darker form than the preceding species, have recently been obtained, and should be included in this discussion. They are very similar indeed to those of the preceding species, but may be separated from them by size and by the nature of the egg mass.

Dimensions of eggs: Length, 1.7 mm.; greatest width, .5 mm.; length of extension of chorion, .2 mm.

These eggs show very clearly the relationship which exists between this species and *Zelus exsanguis* (Stal). While the individual eggs of the two species are very similar, the egg masses themselves are different. While *Zelus exsanguis* (Stal) deposits its eggs in a very compact five- or six-sided mass, *Zelus socius* Uhler produces an elongate mass, of the width of not more than three or four eggs. The material at hand does not permit an investigation into the nature of the cap. It is quite probable that other differences would be found here. A count of a single egg mass of *Zelus socius* Uhler shows it to contain twenty-three eggs.

Pselliopus cinctus (Fabricius).

(Pl. X, figs. 1, 2 and 3.)

This is a beautiful orange-yellow and black species, quite common on the leaves and twigs of trees and bushes, and occasionally found on lower plants, as Chittenden[1] has recorded it as one of the natural enemies of the Colorado potato beetle. No previous detailed description of the eggs of this species has been found, so the description prepared by the writer is given.

Description of egg: Length, 1.7 mm.; greatest width, 1 mm.; least width, just below extension of chorion, .4 mm.; length of extension of chorion, .2 mm. Color, brown; extension of chorion at top of egg, whitish. Somewhat kidney-shaped, wider at base, one side convex, broadly rounded, the other almost straight, very slightly concave; narrowed in region of cap. Extension of chorion at top, membranous, reticulated, whitish, tubelike; cap with central rodlike structure, this extended above tubelike extension of chorion; base of rod provided with reticulated, flaring membrane.

The eggs of this species are laid singly, resting on the broad curve at the lower end of the egg, and attached to their support by a small amount of cement. In the laboratory they were attached to the cheesecloth and wire screen of the cage.

Pselliopus barberi Davis.

I have recently obtained examples of the eggs of this species through the courtesy of Mr. McAtee, of the United States National Museum. A comparison of this egg with that of *Pselliopus cinctus* shows that it is practically identical as far as the more general features of shape and size are concerned. The material is not of such a nature that detailed measurement of parts and critical comparisons of the detailed structures of the cap can be made. It is quite likely that such measurements and comparisons would show distinct differences, however, since these are distinct, though closely related, species.

The eggs of the two last mentioned genera are similar. They have the same general shape and appearance, and each possesses an erect, whitish, reticulated extension of the chorion at the top of the egg. They differ in that the eggs of *Pselliopus* are laid singly, while those of *Zelus* are laid in a mass; and in that the top of the extension of the chorion is turned in to almost cover the cap in *Zelus*, and is not turned in *Pselliopus*. It is quite certain that the eggs of these two genera resemble each other more closely than they do the eggs of any other genus of this, or other subfamilies studied.

Arilus cristatus (Linnæus).

(Pl. XI, figs. 1 and 2.)

This is the insect known as the "Wheel bug," one of the largest and best known of our common reduviids. It is commonly found on weeds, shrubbery, and the lower branches of trees. The egg mass of this insect, judging from the number of references to it, must be known to more people than that of any other reduviid. The reasons for this are that it is a rather common species, that the egg mass is large and conspicuous, and that the entire winter is spent in the egg stage, instead of two or three weeks of the summer as is the case with most reduviids.

Heidemann[5] gives the following descriptive notes concerning the eggs of this species: "The eggs are oval-elongate; dark-brown; chorial processes inside the extended rim, club-shaped."

Howard[7] describes the eggs as follows: "The eggs of the wheel bug look like miniature leather bottles standing on end and in hexagonal clusters, seventy or more in a group, and attached to the bark of trees, on fence rails, or wherever the female happens to be."

Description of egg: Total length, 3 mm.; length to extension of chorion, 2.6 mm.; width, 1.3 mm.; diameter of extension of chorion,

from a little less than greatest width to a little more. Color, brown, darker toward upper end; extension of chorion, whitish; central area of cap, dark brown; appendages of rim of cap, buff. Shape, subcylindrical, rounded at lower end, somewhat narrower at upper end; membranous, reticulated, somewhat flaring extension of chorion at upper end; central disk of cap very slightly raised, outer rim of cap with two sets of filamentous appendages, one turned outward over extension of chorion, the other turned inward over central area of cap.

While the eggs of this species do not resemble those of any other species of the subfamily very closely, yet it must be admitted that they are more like the eggs of *Acholla* and *Sinea* than like those of *Pselliopus* and *Zelus*.

Acholla multispinosa (De Geer).

(Pl. XI, figs. 3 and 4.)

The genus *Acholla* is very closely related to the genus *Sinea*. This relationship is shown not only by the similarity of the adult insects, but also by the similarity of the eggs. This species is a rather common one, being found often on the leaves and branches of trees. While no previous description of the eggs of this insect has been found, there is, nevertheless, an excellent photograph of the egg mass, taken by Mr. Walden,[16] in the "Hemiptera of Connecticut."

Description of egg: Length, 1.7 mm.; width, .7 mm.; diameter of extension of chorion, .9 mm. Color, dark brown, with extension of chorion lighter. Shape, elongate-oval, narrowed at top; extension of chorion at top of egg, horizontal, reticulate, with crenulate margins; cap with an outer, finely and evenly reticulated circular margin; a raised crater, lighter than the rest of the cap; and an inner, darker, flat central disk; crater composed of scales, dark at bases, lighter at apices.

The eggs of this species are laid in compact masses cemented to the twigs of trees. The masses at hand show a count of from thirty-three to forty eggs in each mass.

Sinea diadema (Fabricius).

(Pl. XI, figs. 5 and 6.)

This is one of our most common reduviids, present in almost any open, sunny field, and of possible importance as a beneficial predacious insect. It is a rather small, greyish or brownish, spiny insect, referred to by Ashmead[1] as the "Crowned Soldier Bug." The eggs have been described and figured previously.

The following description of the egg is taken from an article by G. W. Barber[2]:

"Length, 1.3 mm.; width, .6 mm.; diameter of extension of chorion, .8 mm. Color, brown, minutely granulated, somewhat shining; central area of cap, brown; outer rim, brown with minute, regular, white reticulations; extension of the chorion, white with dark lines, brown towards the inner edge; shape, subelliptical, narrowed towards the cap; central area of cap raised, conelike, bluntly rounded at the tip, composed of several scales which fail to meet at the tip; outer rim of the cap flat, with minute, regular reticulations; extension of the chorion on the same plane with the outer rim of the cap in new laid eggs, after hatching or drying bending upwards or downwards, squamous, minutely so towards the inner border, gradually coarser outwards, edge sinuate; chorial process numerous, elongate, club-shaped, within the extension of the chorion."

The eggs of this species were easily obtained by confinement of adults. A number of adults were placed in a rather large cage, and mating pairs were removed from this cage from time to time as observed, and isolated in smaller cages. The eggs were laid freely on the sides of the containers, or on bits of stick and leaves. They are laid in double rows, in groups of from five to twenty-two, the usual number being from eight to twelve. They are attached by means of a cement.

Sinea spinipes (Herrich-Schäffer).

(Pl. XI, figs. 7 and 8.)

This species is very similar in general appearance to the preceding, though it is found in a different habitat. It is taken on the branches and leaves of forest trees most commonly, and appears to be a shade lover. It is not nearly as common a species as the preceding. No previous description of the eggs has been found.

Description of eggs: In size, shape and general appearance very similar to those of *Sinea diadema* (Fabricius). Length, 1.5 mm.; width, .7 mm.; diameter of extension of chorion, .9 mm. Color, brown; extension of chorion, buff, darker near rim of cap; central area of cap, brown, with central spine darker; membrane around rim of cap, darker. Shape elongate-oval, slightly narrowed towards cap; extension of chorion at top of egg nearly horizontal, reticulated; margin, irregular, much incised. Central area of cap occupied by disk, erect spine in center; a dark membrane around rim of cap, membrane divided into many very delicate, filamentous, erect appendages.

The eggs of this species were obtained only in the laboratory, and could be secured easily by confinement of adults. The eggs are laid

in a rather irregular, double row, being attached to their support by a very small amount of cement. There may be from three to fourteen eggs in one of these groups.

The eggs of the last three species considered are very similar. They resemble each other in size, shape, and general appearance. Those of *Acholla multispinosa* (De Geer) differ from those of either species of *Sinea* in that they are laid in a compact mass, rather than in a double row. The eggs of *Sinea diadema* (Fabricius) differ from those of *Sinea spinipes* (Herrich-Schäffer) in that the extension of the chorion is lighter in color and not so deeply incised in *Sinea diadema* (Fabricius), and in that the scales of the cap are more numerous and conspicuous, and inclined toward the center of the egg in *Sinea diadema* (Fabricius), while they are fewer in number, more delicate and inconspicuous, and stand nearly erect in *Sinea spinipes* (Herrich-Schäffer).

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PLATE IX.

FIG. 1. Egg of *Reduvius personatus* (Linnæus).
FIG. 2. Egg of *Triatoma sanguisuga* (Le Conte), (after Miss Kimball).
FIG. 3. Egg of *Melanolestes picipes* (Herrich-Schäffer), viewed from side.
FIG. 4. Same, viewed from above, twice scale of 3.
FIG. 5. Egg of *Rasahus biguttatus* (Say), viewed from side.
FIG. 6. Same, viewed from above, twice scale of 5.

PLATE IX.

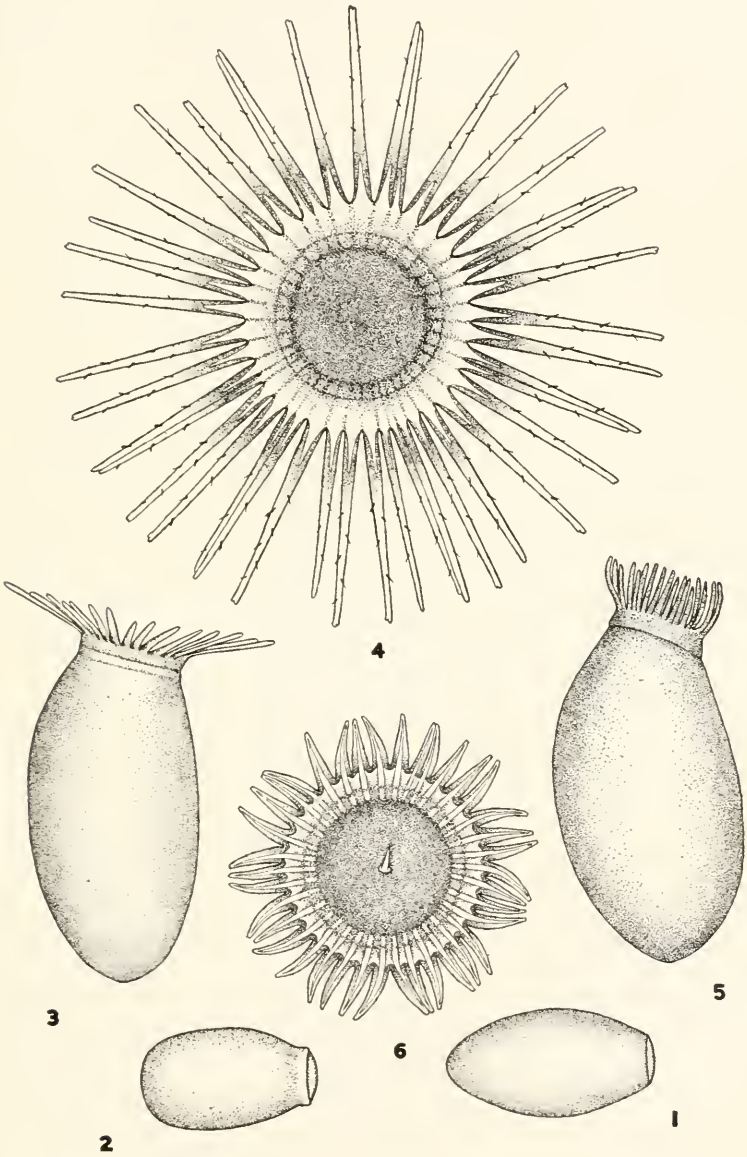
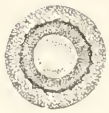


PLATE X.

FIG. 1. Egg of *Pselliopus cinctus* (Fabricius), viewed from side.
FIG. 2. Same, viewed from above, twice scale of 1.
FIG. 3. Same, cap removed in hatching, twice scale of 1.
FIG. 4. Egg of *Oncocephalus apiculatus* Reuter, viewed from side.
FIG. 5. Egg of *Zelus exsanguis* (Stal), viewed from side.
FIG. 6. Same, viewed from above, twice scale of 5.
FIG. 7. Same, cap removed in hatching, twice scale of 5.
FIG. 8. Egg of *Zelus socius* (Uhler), viewed from side.
FIG. 9. Egg of *Apiomerus spissipes* (Say), viewed from side.
FIG. 10. Egg of *Emesaya brevipennis* (Say), viewed from side.
FIG. 11. Egg of *Metapterus fraternus* (Say), viewed from side.
FIG. 12. Egg of *Apiomerus spissipes* (Say), viewed from above, twice scale of 9.

PLATE X.

1



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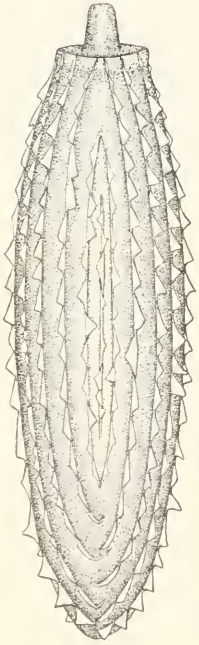

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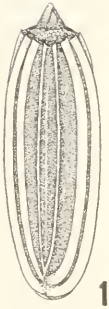

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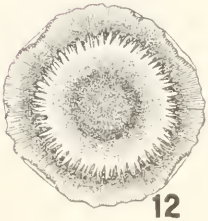

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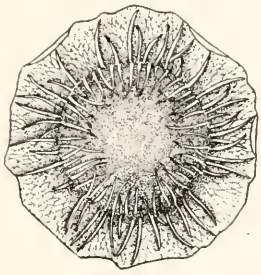
PLATE XI.

FIG. 1. Egg of *Arilus cristatus* (Linnæus), viewed from side.
FIG. 2. Same, viewed from above, twice scale of 1.
FIG. 3. Egg of *Acholla multispinosa* (De Geer), viewed from side.
FIG. 4. Same, viewed from above, twice scale of 3.
FIG. 5. Egg of *Sinea diadema* (Fabricius), viewed from side.
FIG. 6. Same, viewed from above, twice scale of 5.
FIG. 7. Egg of *Sinea spinipes* (Herrich-Schäffer), viewed from side.
FIG. 8. Same, viewed from above, twice scale of 7.

PLATE XI.

1



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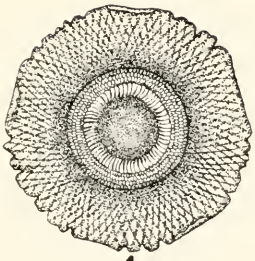

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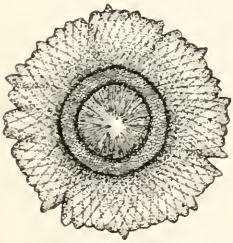

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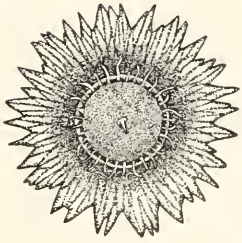

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SCIENCE BULLETIN

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The Nature, Origin and Significance of Pigment in Embryos of *Amblystoma*.

HERVEY S. FARIS, Department of Anatomy.

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INTRODUCTION.

Although various workers have observed a yellowish-brown pigment scattered through the tissues of amphibian embryos, the writer has been unable to find any satisfactory or comprehensive report in literature concerning it. The purpose of this work is to describe the nature, origin and significance of this pigment in the myotomes and central nervous system of embryos of *Amblystoma*.

For valuable suggestions and criticisms the author is indebted to Professors G. E. Coghill and H. C. Tracy. Furthermore, through grants made to them by the Research Committee of the Graduate School of the University, this work has indirectly profited in a considerable degree.

MATERIAL AND METHODS.

This study has been made chiefly on *Amblystoma microstomun*. The stages of development selected for observation have included those described by Coghill (1916, p. 163) as, (1) embryos that move in response to an electric current, but not in response to tactile

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stimulation with a hair or other chemically inactive structure, designated the nonmotile stage; (2) embryos taken very soon after there is a perceptible movement in response to tactile stimulation, designated early flexure stage; (3) embryos that move the trunk into a coiled condition, designated the coil stage; (4) embryos that move to the right and left simultaneously in different parts of the body in a sinuous fashion, and have just acquired the power of locomotion, designated the early swimming stage. In addition observations were made on older embryos, the ages of which are indicated by the number of days from the time they began to swim.

Serial sections have been used extensively in these observations. The fixing fluids that have been employed are Bouin's, corrosive sublimate and acetic acid, Van Gehuchten's, Zenker's without acetic acid, Orth's, Kleinenberg's, and formalin solutions. The first two are found to be especially suited to this purpose. Erythrosin and toluidin blue, alum-carmine and Lyon's blue, alum-carmine, hæmatoxylin and orange G, neutral red, and neutral red followed by Janus green, have been used, but alum-carmine has proved to be best adapted for study of pigment in the central nervous system. Hæmatoxylin and orange G show very satisfactorily the relations of the larger structures of the muscle cell, such as the nuclei, yolk globules, and the large masses of pigment which will be described further on in the paper. Neutral red and Janus green are superior for the demonstration of the finer structures of the cell, such as pigment granules, neurofibrils and myofibrils.

Teased preparations and frozen sections of fresh tissue have been used for the study of the nature and origin of the pigment. Preparations have been made by carefully dissecting various tissues such as post-auditory myotomes, central nervous system, cutis, and undifferentiated mesenchyme. These tissues were placed in various stains for different lengths of time. After staining, the larger tissues were teased out on a slide in a drop of the stain and then covered with a cover-glass. By gentle pressure on the cover-glass the tissue was spread thin enough to permit examination under oil immersion objectives. Thinner or smaller pieces of tissue were, after staining, mounted in a hanging drop. Care was taken to use a small drop which would permit the use of oil immersion objectives. The stains used in these preparations were neutral red, Janus green, Nile blue, methylene blue, Sharlach R and Sudan III. Unstained preparations were also studied.

OBSERVATIONS.

A. PIGMENT IN MYOTOMES.

The relation of the pigment to other structures in the cell and its distribution through the myotomes has been studied in corresponding myotomes of the different stages. Serial sections in the frontal plane have been used almost exclusively for these observations.

1. Nonmotile Stage.

(Plate XIII.)

The cells in the dorsal portion of the third myotome are not formed into functional muscle fibers, but are radially arranged about the central part. Dorsoventrally there is a progressive change to the longitudinal arrangement of muscle cells containing two or three nuclei. Myofibrils are present in the ventral portion, although obscured by the yolk globules, which are very abundant. In the dorsal portion the pigment occurs in the form of fine granules in the central part of the myotome. These granules are located about the central ends of the nuclei in the radially arranged cells, and around yolk globules. Farther ventrally, the pigment occurs in the middle third of the myotome longitudinally. The pigment granules occur about the nuclei, or the yolk globules, or in strands between these structures. They are more abundant in the ventral portion, and not only occur as fine granules, but also in masses of closely adhering, or segregated, granules. These masses of pigment granules are here termed "pigment bodies." They occur in most instances at one pole of a nucleus. While the pigment bodies appear homogeneous under low magnification, under oil immersion they are resolved into aggregations of granules.

There are very few pigment bodies in the upper third of the myotome. The number increases dorsoventrally through the ventral two-thirds of the myotome. This is illustrated in Figure 1*a*, which is an optical projection of the third myotome, as viewed from the lateral side, showing the number and location of the pigment bodies. The total number of pigment bodies is sixty. The average diameter of thirty of the largest of the pigment bodies is 6.36 micra.

The tenth myotome is less differentiated than the third. The cells in the dorsal region retain their embryonic type and radial arrangement further ventrally than in the third myotome. The nuclei in this more dorsal region are nearly round. In the ventral portion the cells become definitely oriented longitudinally, parallel to the long axis of the myotome. In this region the nuclei are slightly

elongated. No myofibrils can be observed. The pigment through the central portion occurs in the same relative position as in the corresponding region of the third myotome. There are only eighteen pigment bodies in the entire myotome. (Fig. 1*b*.)

The eighteenth myotome is still less differentiated than the tenth. The radial arrangement of the cells about the central portion exists throughout the whole myotome. There are apparently no functional muscle cells. The structure of such a myotome is illustrated in Plate XIII. There are no pigment bodies present. The pigment granules are distributed through the central portion of the myotome about the yolk globules, and around the nuclei they are particularly abundant at the ends which are directed toward the center of the myotome.

2. EARLY FLEXURE STAGE.

(Figure II.)

The third myotome in this stage is more differentiated than the corresponding myotome of the nonmotile stage. The relatively undifferentiated dorsal portion is less in extent. The myofibrils can now be perceived in the middle part of the myotome dorsoventrally in regions where the yolk globules are not too numerous. The differentiation has increased over that of the nonmotile embryo as shown by the elongation of the nuclei and muscle cells, and by the increase of myofibrils. There appears to be relatively less yolk. There is an increase in the amount of pigment. The pigment bodies number 152 in the entire myotome. (Fig. II*a*.) The majority are located near the ends of nuclei. The average mean diameter of thirty of the largest is 8.06 micra.

The tenth myotome has differentiated further than the corresponding myotome of the younger stage. It is less differentiated than the third myotome of the stage under consideration as described above. The undifferentiated cells in the dorsal region gradually change to the longitudinal arrangement in the ventral portion, where the outlines of the cells, the position of the nuclei, and the presence of myofibrils show that the muscle is functional in this region. The yolk globules are more numerous at the ends of the myotomes than in the central part. The pigment granules and bodies have the same general arrangement as in the third myotome. Less pigment is present than in the third myotome, but more than in the corresponding myotome of the earlier stage. This myotome in the early flexure stage contains seventy-one pigment bodies. (Fig. II*b*.) The average mean diameter of thirty of the largest is 7.65 micra.

The eighteenth myotome is more differentiated than the corresponding myotome of the nonmotile stage. In the dorsal third the cells occur in a relatively radial arrangement about the central part of the myotome. The cell boundaries are still indistinct, a condition which characterizes the whole of the corresponding myotome of the younger embryo. There is a gradual transition in this myotome from the radial arrangement of the cells in the dorsal third to the elongated longitudinally directed muscle cells which are characteristic of the ventral third of the myotome. These muscle cells are multinucleated, and each extends the length of the myotome. The pigment in the dorsal portion is distributed in the central part of the myotome as shown in Plate XIII. In the ventral, differentiated portion of the myotome the pigment granules and bodies occur in the central third of the cells. There are only twelve small pigment bodies in the entire myotome. (Fig. IIc.)

3. Coil Stage.

(Figure III.)

The third myotome is further differentiated than the corresponding myotomes of the next younger stage, as shown by the increase in the amount of functional muscle in the dorsal portion, the increase in the number of myofibrils, and the elongation of the myotome. There appears to be relatively less yolk. The increase in the amount of pigment is slight. There are 161 pigment bodies present. The average mean diameter of thirty of the largest bodies is 8.12 micra.

The tenth myotome is less differentiated than the third, but is more differentiated than the tenth myotome in the earlier stage. In the dorsal part, the nuclei are crowded together in a transitional stage between the radial arrangement and the elongated cell types. The ventral half of the myotome contains approximately eighty-five per cent of the pigment bodies, which number 150. (Fig. III*b*.) Thirty of the largest of these bodies average 8.16 micra in diameter.

The eighteenth myotome shows a considerable increase in development over the corresponding myotome of the early flexure and nonmotile stages. The middle and ventral portions are differentiated into functional muscle. The pigment granules and bodies have the same arrangement throughout the middle third of the myotome longitudinally, and through the myotome dorsoventrally, as described for the tenth myotome of this stage. There are thirty-five pigment bodies located in the ventral portion in the middle third of the myotome. The average mean diameter of thirty of the largest is 5.9 micra.

4. EARLY SWIMMING STAGE.

(Fig. IV, and Plates XII, XIV.)

The third myotome shows the greatest differentiation of any myotome studied. (Pl. XII.) Cross striations are plainly visible. There is, apparently, a decrease in the amount of yolk as compared with the corresponding myotome of the coil stage. The yolk globules are smaller than in the corresponding myotomes of the younger stages. The number of pigment bodies has increased to 226 as compared to 161 in the coil reaction stage, 152 in the early flexure stage, and 60 in the nonmotile stage. The average mean diameter of thirty of the largest is 8.65 micra. The bodies are typically arranged in the central portion of the myotome at or near the ends of the nuclei.

The tenth myotome is further differentiated than the corresponding myotomes of the earlier stages. There is, apparently, less yolk and a decided increase in the number of pigment bodies. In this myotome there are 364 pigment bodies as compared to 150 in the coil stage, 71 in the early flexure stage, and 18 in the nonmotile stage. The average mean diameter of thirty of the largest is 8.24 micra. The dorsal part of the myotome has many granules, but few bodies. In the ventral part the bodies are numerous, with a relatively smaller number of granules; a similar arrangement prevails in the adjacent myotomes of that region. As the pigment bodies increase in number and size from the dorsal toward the ventral part of the myotome the granules appear to decrease in number until there are only a few in the ventral portion of the myotome, where the pigment bodies become correspondingly more numerous.

A section through the middle of the myotome in frontal plane shows that the nuclei are arranged in three zones. In the outer zones the nuclei are arranged in a single definite row transversely, whereas, in the middle zone they are scattered throughout the middle third of the myotome, but separated from those of the outer zones by a characteristic space, void of nuclei. (Pl. XIV.) The pigment bodies occur at or near the ends of the nuclei of the middle zone and at the inner ends of the nuclei of the outer zones.

The pigment granules are particularly abundant throughout the region between the two outer zones, while the end portions of the myotome contain few granules.

The positions of eighty-one pigment bodies were noted. Fifty-eight, or 71.6 per cent, were at the ends of the nuclei; fifteen, or 18.5 per cent, were at the sides of nuclei, and eight, or 9.9 per cent, of the bodies were located some distance from nuclei. That 9.9 per

cent of the bodies were not in the immediate vicinity of a nucleus may be due to their having been separated from nuclei by the sectioning of the tissues.

The eighteenth myotome is less differentiated than the tenth, as the dorsal portion shows the radial arrangement for a short distance. There are 195 pigment bodies in this myotome (Fig. IV*c*) as compared to thirty-five in the coil stage, twelve in the early flexure stage, and none in the nonmotile stage. The average mean diameter of thirty of the largest is 6.23 micra. The distribution of the pigment bodies is the same as described in other myotomes.

The twenty-fourth myotome shows only a slight degree of differentiation. The pigment granules are distributed around nuclei and yolk globules, similar to the distribution described in the eighteenth myotome of the early flexure stage. In the entire myotome there are only five pigment bodies. These are located within a narrow zone in the middle third. (Fig. IV*d*.)

5. Comparative Summary.

The numerical relation of the pigment bodies in the third, tenth, eighteenth, and twenty-fourth myotomes is shown in Table I.

Table I.—Number of pigment bodies in myotomes.

Stages.	3d	10th	18th	24th
Nonmotile	60	18	....	....
Early flexure	152	71	12	....
Coil	161	150	35	....
Early swimming	226	365	195	5

In the third myotome the number of bodies increases from 60 in nonmotile stage to 226 in the early swimming stage; in the tenth myotome the increase is from 18 to 365; in the eighteenth myotome the number increases from zero to 195, while in the twenty-fourth myotome the increase is from zero to five. The increase in number appears to be slightly retarded in the third myotome after the early flexure stage. This is probably due to the growth, in this region, of the pronephros which prevents the full development of the third myotome, and is the probable explanation of the fact that the third myotome has in the early swimming stage fewer pigment bodies than the tenth myotome.

Table II shows the relative growth in size of the pigment bodies in corresponding myotomes of the four stages.

TABLE II.

THIRD MYOTOME.

	Dorsal	Middle	Ventral	Average
Nonmotile	5.55	6.44	7.16	6.36
Early flexure	6.09	8.79	9.4	8.06
Coil	6.7	8.22	9.45	8.12
Early swimming	6.96	8.06	10.95	8.65
Average	6.32	7.87	9.24	7.81

TENTH MYOTOME.

	Dorsal	Middle	Ventral	Average
Nonmotile	....	....	....	....
Early flexure	6.29	8.19	8.48	7.65
Coil	6.68	8.82	8.99	8.16
Early swimming	6.7	8.83	9.2	8.24
Average	6.58	8.61	8.89	8.02

EIGHTEENTH MYOTOME.

	Dorsal	Middle	Ventral	Average
Nonmotile	....	....	....	....
Early flexure	....	....	....	....
Coil	5.04	6.16	6.5	5.9
Early swimming	5.2	6.49	7.02	6.23
Average	5.12	6.32	6.76	6.06

In computations for Table II ten of the largest pigment bodies were selected for measurement in each of the following portions of the myotome: (1) dorsal, (2) middle, and (3) ventral. The greatest and least diameters were measured in micra and the mean taken. This table shows that there is an increase in size of the pigment bodies from the youngest to the oldest stage, and from the dorsal to the ventral portion of a given myotome. In the middle portion of the third myotome there appears to be a decrease in size from the early flexure stage to the early swimming stage. This may be due to the growth of the pronephros in this region, which by the mechanical factors involved introduces irregularities in development such as shifting of the position of the myotome.

The increase in size in the dorsal part of the third myotome from early flexure to the early swimming stage is from 5.55 micra to 6.96 micra, an increase of 1.4 micra, or twenty-five per cent. In the middle portion of the myotome the increase is from 6.44 to 8.06 micra, which amounts to 1.62 micra, or twenty-five per cent. In the ventral part the increase is 3.79 micra, or fifty per cent. The bodies increase in size dorsoventrally. In the early flexure stage this increase is from 5.55 micra to 7.16, which amounts to approximately thirty per cent. In the early swimming stage the increase is 3.99 micra, or nearly sixty per cent.

6. CONCLUSIONS.

The results of the study of the serial sections show that, (1) the pigment is found chiefly in the central portion of the myotome; (2) the pigment granules are situated about nuclei and yolk globules and extend in strands between these structures; (3) the pigment bodies increase both in number and size from the dorsal to the ventral portion of the myotome; (4) the pigment bodies in corresponding myotomes increase both in number and size with the age of the embryo, at least up to the early swimming stage; (5) the amount of pigment increases as the yolk decreases; and (6) the amount of the pigment varies directly with the degree of differentiation and the development of function.

B. PIGMENT IN THE CENTRAL NERVOUS SYSTEM.

(Fig. V.)

Since there proved to be a distinct correlation between the increase in pigment and the degree of differentiation in the myotomes, the study was extended to the distribution of the pigment in the central nervous system as related to regions of differentiation and proliferation.

Although observations have been made on a large number of embryos, type specimens were selected for a more exhaustive study. The embryos used were in my series designated, No. 304, nonmotile; No. 309, early flexure; No. 308, coil; and No. 302, early swimming. They were all fixed in Bouin's fluid, sectioned transversely five micra in thickness, and stained with carmine. With a projecting apparatus, magnifying two hundred diameters, sketches were made of every fourth section of the nervous system, beginning at the anterior portion of the forebrain. Outlines of the areas of abundant pigmentation were sketched in.

These drawings were continued caudad as far as the pigment could be seen through the projecting apparatus. The drawings were compared with reference to the general distribution of pigment and this comparison was supplemented and checked by direct study of the sections with the microscope. It should be noted that fine granules of pigment occur throughout the central nervous system, but that the regions in which the pigment was especially abundant were indicated by the projection apparatus. The visibility of the pigment through this apparatus thus became a standard for quantitative estimates.

The distribution of the pigment was noted particularly in the rhombencephalon and spinal cord. Different levels were selected for descriptive purposes in order to compare more easily the distribution of pigment in the different stages and to differentiating areas that have been previously described by Coghill (1914, 1916).

Before consideration of these areas and their relations to differentiation, it should be noted that the series of sketches of the sections mentioned above, as well as Figure VIII, were made before areas of differentiation had been taken into account by the author.

1. RHOMBENCEPHALON.

In the rhombencephalon the pigment is located towards the surface in lateral and ventrolateral areas. This occurs throughout the Rhombencephalon and continues caudad in the spinal cord in lateral areas.

(*a*) *At the Level of the Root of the Trigeminal Nerve.*

In the *nonmotile* stage the rhombencephalon still retains the upright position dorsoventrally. The cells of the inner portion of the rhombencephalon next to the ventricle are epithelial in appearance and arrangement. This structure prevails nearly to the outside of the brain. Here in this region are a few cells that have already differentiated into nerve cells. Their relation to the trigeminal nerve has been described by Coghill (1916, Fig. 20). This lateral and ventrolateral region is the area of abundant pigmentation shown by Figure V, row I and column 1. There is a great abundance of pigment about nuclei and yolk globules. It is most abundant towards the external surface of the brain. There is some pigment along the ventricular surface which probably has been retained from the earlier ectodermal position. There is a large clump of pigment next to the ventricle in the floor plate cells.

In the *early flexure* stage the rhombencephalon has differentiated further than in the nonmotile stage, although there has been only a slight broadening. The area of differentiating neuroblasts is slightly wider in extent. (See Coghill, 1916; Fig. 21.) The pigment is distributed through this area as shown by Figure V, row II, and column 1. The pigment area has increased dorsoventrally, and there appears to be a slight increase in amount of pigment. The largest and heaviest groups of pigment occur toward the outside. In the type specimen from which the drawings were made, there is more pigment immediately along the ventricular border of the

rhombencephalon than in the other specimens. The pigment in the floor plate cells is massed together in the central end of the cells adjacent to the ventricle.

In the *coil* stage the rhombencephalon has broadened so that the areas of differentiation that were lateral in the younger stages are now distinctly lateral and ventrolateral. The cells adjacent to the ventricle are epithelial in appearance and arrangement as in the younger stages, but this epithelial area is less in extent. In this proliferating area there is no increase in the amount of pigment. In the lateral differentiating area there is an increase in the number of neuroblasts. (See Coghill, 1916; Fig. 35.) As the area of differentiation has increased so has the area that contains the pigment in abundance. The pigment extends further toward the ventricle, although most abundant in the outer cells. This is shown in Figure V, row III, and column 1. There appears to be slightly more pigment than in the younger stages at the corresponding level. There is less pigment bordering the ventricle, except the large mass in the floor plate cells.

In the *early swimming* stage there is a further differentiation in this stage over that of the coiled reaction stage. The fiber tracts and differentiating neuroblasts are easily perceived. The pigment is more abundant in the lateral and ventrolateral areas as described in the younger stages. (See Coghill, 1916, Figs. 51-54.) The pigment area has increased in size, however, and is now wider and extends over half the distance to the ventricle. Outside of this area the pigment is not particularly abundant, except in the floor-plate cells, where there is a large mass adjacent to the ventricle. In the lateral and ventrolateral areas the pigment occurs as fine granules. Sometimes under low magnification, some of the pigment appears to be in irregular, stringy masses, but higher magnifications show that they consist of closely adhering granules.

(b) At the Level of the Roots of the Facial and Glossopharyngeal Nerves.

The areas of abundant pigmentation as described at the level of the root of the N. V. are continuous in the lateral and ventrolateral regions from the root of the N. V. through the levels of the roots of the NN. VII and IX into the lateral areas of the spinal cord.

In the *nonmotile* stage there is apparently less pigment at the level of the seventh than at the level of the fifth nerve and approxi-

mately the same as at the level of the ninth nerve. At the level of the ninth nerve the pigment is in more lateral areas.

In the *early flexure* stage there is an apparent increase of pigment at the level of the seventh and ninth nerves as compared to that of the nonmotile stage. This is most marked at the level of the ninth nerve, which follows closely the increase of differentiation as shown by widening of the rhombencephalon at this level. The extent of the pigment increases correspondingly with the larger area of differentiation. The change in shape of the rhombencephalon at these levels and the increase in extent and amount of pigment is shown in Figure V, rows I and II, and columns 2 and 3.

In the *coil* stage there is a further widening of the rhombencephalon, with an increase in differentiation, and likewise an increase in amount and extent of the pigment. In this stage there seems to be approximately the same amount of pigment at the levels of the seventh and ninth nerves as at the level of the fifth nerve. No increase in amount of pigment could be noted in the proliferating areas next to the ventricle. The distribution of the pigment is shown by Figure V, row III, and columns 2 and 3.

In the *early swimming* stage the differentiating areas are wider than in the younger stages, and in a similar way the area of pigmentation is wider and extends more toward the ventricle. The pigmentation is heavier and more pigment occurs in clumps. The greatest amount occurs near the outer edge of the brain. There is approximately the same amount of pigment at these levels as at the level of the fifth nerve. There is no increase in pigment along the border of the ventricle.

2. Spinal Cord.

The distribution of the pigment in the spinal cord is shown by the projection apparatus to be in lateral areas. The pigment can be traced by this method to the level of the fifth myotome in the nonmotile stage. In the early flexure stage it extends to the level of the eighth myotome; in the coiled reaction stage to the level of the ninth myotome, and in the early swimming stage to the level of the eleventh myotome.

At the level of the third myotome in the nonmotile stage the pigment occurs in the lateral areas as shown in Figure V, row 1, column 4. This corresponds to the differentiating area described by Coghill (1914; Fig. 28). In the early flexure stage there is an increase in amount of pigment in the lateral areas. This is shown in Figure VIII, row II, and column 4. There is no increase in pigment in the

proliferating areas next to the central canal. (See Coghill, 1914; Fig. 29.)

In the coil stage the pigment has increased in extent, as shown in Figure VIII, row III, and column 4. In the early swimming stage there is additional increase in pigment in the differentiating areas as opposed to lack of increase in the proliferating areas. (Coghill, 1914; Figs. 30 and 31.)

This increase in pigment at the level of the third myotome as the embryo develops is distinct. It is even more marked at the level of the eighth myotome. There was no pigment area visible at this level in the nonmotile embryo. In the early flexure stage there was a small amount visible in the lateral area. In the coil stage this area is larger and contains more pigment. In the early swimming stage the pigment has increased in amount and occurs in the area of most extensive differentiation. (See Coghill, 1914; Figs. 32 to 35.)

3. Comparative Summary of Pigmented Regions in the Nervous System.

The definite increase in the amount of pigment could not be measured exactly. The areas of abundant pigmentation, however, increase in size at each of the levels of the neutral nervous system noted in the description. The comparative size of these areas is shown roughly in Figure V. There are no perceptible clumps in the youngest stages, and only occasionally do they occur in the older stages. The amount of pigment in the nonmotile embryo decreases gradually through the rhombencephalon and rostral part of the spinal cord. In the early flexure and coil embryos, the pigmentation increases in the rhombencephalon at the level of the root of N. V., but seems to increase more rapidly at the levels of the roots of NN. VII and IX, until in the early swimming embryo the amount is approximately the same in the lateral and ventrolateral areas at the different levels under consideration.

The increase in amount of pigment and size of areas of abundant pigmentation is more marked in the spinal cord than in the rhombencephalon. Especially is this noticeable at the level of the eighth myotome. The areas of pigmentation can be traced farther caudad as the embryo increases in age.

It is obvious that the areas of abundant pigmentation increase in size with the growth of the embryo, and that there is no increase in pigment in the proliferating areas adjacent to the ventricle. This is true in both the rhombencephalon and spinal cord.

It should be noted that in the youngest stages studied certain cells contain pigment. Coghill (1914) noted that the Rohon-Beard cells in the spinal cord contain masses of pigment. These cells, together with a few motor cells and certain cells in the floor plate, differentiate early, and the pigment, which is probably formed during their differentiation period, is retained in the cytoplasm during the four physiological stages studied.

4. Conclusions.

From the facts as observed one must conclude that, (1) differentiating areas are areas of most abundant pigmentation; (2) the amount of pigment increases as differentiation proceeds; (3) proliferation is not accompanied by an increase of pigmentation; and (4) prior to the ingrowth of blood vessels the amout of pigment is an index of the degree of differentiation in the central nervous system.

C. ORIGIN OF PIGMENT.

The study of the formation of the pigment in *Amblystoma* embryos has been performed primarily upon fresh preparations of living tissue. Observations have been made not only on the muscle and nervous systems, but also upon preparations from epidermis and undifferentiated mesenchyme. Dissected tissues, both stained and unstained, were mounted on slides and gently mashed under a cover-glass, or placed in hanging drops. Epithelial cells of the skin and undifferentiated mesenchyme cells were mostly used for this work because better preparations can be made from them than from the central nervous system and myotomes; the character of the pigment is the same in either case.

Preparations were kept under observation from one-half to several hours. The longest observations were not continuous, but particular fields were observed at frequent intervals for from ten to fifteen hours.

1. Unstained Preparations.

Preparations of undifferentiated entodermal cells are better adapted for study of relations of pigment and yolk. The yolk globules vary greatly in size from twenty to thirty micra in diameter to fine globules almost as small as pigment granules. The large globules are motionless. Very frequently the smaller globules exhibit movement which is probably Brownian movement. The movement is more evident in some fields than others. It is not appreciably increased by the use of a warming stage.

Pigment granules occur free in the preparations apart from yolk globules or appear on the surface of globules. Few granules occur on large yolk globules. Medium sized globules frequently have on their surfaces two or more pigment granules. Occasionally a small globule is almost covered with pigment granules. The majority of yolk globules are free of pigment granules.

Pigment granules that are on yolk globules may or may not exhibit movement. Those on the larger globules either do not exhibit motion, or are seen to be gliding over the surface. The pigment granules on medium-sized globules move rapidly over the surface. Pigment granules on some of the smallest globules appear to be more agitated. This movement appears to be increased because the yolk globule is itself in motion. In spite of the moving of the yolk globule and of the pigment granules over its surface, it is very seldom that a pigment granule is seen to detach itself from the yolk globule. The pigment granules that are free in the preparation exhibit movement, which is considered to be Brownian movement. Pigment granules are seen to move from place to place, but this may have been due to the streaming in the preparation. The movement of free granules in certain cells, for example young epithelial cells of the skin, have been seen to move not only by Brownian movement, but also by a jerky vibratory movement which is thought to be similar to the characteristic movement as described by Smith (1920) for pigment granules in embryo chick's eye.

There is no particular change in relation of the pigment to the yolk globules from nonmotile to early swimming stage.

The pigment granules that are associated with yolk are yellow-brownish to brown in color. In the older specimen, preparations were made that contained pigment granules from chromatophores of the skin. These are larger in size and much darker in color than the pigment granules under consideration. There are no pigment granules in the nuclei.

In addition to the pigment granules found in the preparations, there are occasionally fine colorless granules situated on yolk globules. They do not occur in abundance, and it is rare to find many in the same field.

2. Neutral Red Preparations.

Preparations were made of fresh tissue from *Amblystoma* which had been grown in dilute solutions of neutral red, or of dissected portions of an embryo that were stained in dilute solutions of the dye. After killing the embryo, the tissue was dissected with fine

needles, by aid of a binocular dissecting microscope, and placed in stain diluted by physiological salt solution. Different tissues, such as skin subcutaneous tissue, muscle, and nervous system and of yolk, were placed in solutions of neutral red. The dilution varied from 1:5,000 dilution to 1:40,000. A dilution of approximately 1:20,000 was generally used. The tissues were stained from ten minutes to four hours. Tissues were either mounted on a slide in a solution of the dye, and covered with a cover glass or mounted in a hanging drop. The cover-glass method is preferable for thick tissues such as myotomes, as the weight of the cover-glass spreads the tissue sufficiently to permit light to penetrate the preparation. The hanging drops are preferable for younger stages, particularly for cells of the skin and subcutaneous tissue, as the normal condition of the rounded form of the cells can be maintained and observed. The tissue fragment and the hanging drop in which it was suspended were both necessarily small in order to permit the use of oil immersion lens through all the preparation. Hanging drop preparations are particularly adapted for this work, as air is kept out by sealing the edges of the cover-glass with a mixture of paraffin and vaseline, which permits observations for a number of hours.

Embryos of different ages were grown in solutions of neutral red of dilutions from 1:50,000 to 1:200,000. The more dilute solutions were generally used, as the embryos take up the dye very readily. Tissue preparations were made from embryos that had lived two days or longer in the neutral red. The results of the intravitam staining were the same as staining fresh preparations of dissected portions.

Observations upon these intravitam preparations of the early swimming stage show the same general arrangement of pigment bodies in the myotomes as found in serial sections, namely, throughout the middle third of the myotome. The pigment granules in muscle cells are located about nuclei and yolk globules and in strands between these structures or free in the cytoplasm. Although the pigment granules were visible in the central nervous system, it was impossible to determine their distribution in the fresh preparations.

In the youngest specimens studied, the epithelial cells of the skin contain much yolk in form of spherules or globules. In these cells the pigment occurs most abundant about the yolk globules in the periphery. The nucleus contains no pigment.

The differential staining of the yolk globules with neutral red and of the relations of the pigment to them is most striking. In prepara-

tions containing groups of yolk globules, the majority of the yolk globules do not stain. The larger globules do not react to the dye as readily as the medium sized and smaller ones. Yolk globules that stain are colored light pink to light red. Occasionally a portion of a stained globule is stained a deeper red. This deeper stained portion often has the appearance of an excrescence or of a ridge usually crescent shaped on the globule. Gradations in staining reactions of yolk globules also occur, as well as differential staining of portions of the same globules.

There are occasionally granules on yolk globules that react to the dye. These are stained differentially from bright red to a deep red. These granules no doubt are identical with the "beta" bodies as described by Coghill (1915). These granules glide over the surface of yolk globules. Some of the dark-stained granules have been observed to leave a globule, and when free in the preparation exhibit the Brownian movement, and they cannot be distinguished from the free-pigment granules. The pigment granules, whether free or in a group, appear to stain a very deep red.

While the majority of the observations were performed on preparations from undifferentiated entodermal cells, these observations were corroborated with evidences found in preparations of other tissue cells. Epithelial cells from skin of young embryos of nonmotile stage show the same differential staining of yolk and of neutral red granules with reference to pigment. In such a cell, the structures are more compact. A larger percentage of yolk stains. There are more smaller globules that have neutral red granules or pigment granules exhibiting a vibratory gliding motion over the surface. The nucleus does not stain, or contain pigment granules.

The minute colorless granules observed in unstained preparations were not seen in neutral red preparations. Melanin pigment granules from chromatophores of the older stages sometimes were included in the preparations. The melanin granules from chromatophores are larger and do not stain with neutral red. They are dark brown in color. Small yolk globules slightly larger than pigment granules occur in some fields and do not react with the neutral red.

3. Janus Green Preparations.

In preparations stained by Janus green the yolk globules do not stain readily. The small number that react to the stain are light greenish-blue in color. Upon the surface of some of these yolk globules are granules few in number that are stained by the Janus

green. These vary in form and location. They may appear singly or as a row or ridge of fine granules. These granules, no doubt, are the "alpha" granules described by Coghill (1915). Granules were observed to leave the globules, but not frequently. When they do leave they cannot be distinguished from similarly stained granules in the preparations which are probably mitochondria, since Janus green is a specific stain for mitochondria. Mitochondria are not present in large numbers, nor were they observed to develop into pigment granules. The pigment granules are not stained by Janus green.

In some preparations there are occasionally globules that have a pinkish tinge, in whole or in part. This probably is due to reduction of the dye as noted by Coghill (1915). Cowdry (1914) also mentions that reduction of Janus green produces such a change. The change of granules from greenish blue to the pinkish purple color has not been observed. (Coghill 1915.)

4. NEUTRAL RED AND JANUS GREEN PREPARATIONS.

The use of neutral red and Janus green as a double stain is in the main in agreement with Coghill (1915). The globules stained with green are the same as those which he called "alpha" globules. In a preparation of yolk in which the "beta" globules are most numerous I saw a group of four "alpha" globules that were pink in part. Three of them had green-colored granules on the surface which were probably "alpha" granules. Further observation showed that the pink portion of one of them was increased. This may have been due to turning of the globules, but may have been caused by reduction of the dye. During the period under observation, none of the granules were seen to leave globules. However, the dark red "beta" granules observed on the red-colored "beta" globules have been seen to leave the globules, and when this occurred they could not be distinguished from the pigment granules free in the preparation.

Sometimes preparations from skin of the younger stages would show very well the action of the two dyes. In a hanging drop, epithelial cells retain a rounded or oval shape; the nucleus is excentrically placed; the yolk globules are very numerous, and pigment granules scattered through the cytoplasm. The nucleus does not stain. There are more "beta" globules. Some of the "beta" globules have "beta" granules on their surface. Excrescences occur on some "beta" globules. The dark colored "beta" granules cannot be distinguished from the pigment granules free in the cytoplasm. The

pigment granules stain a deep red. They are more numerous in the periphery of the cell. Brownian movement occurs to a limited degree. "Alpha" granules were seen, but are not numerous. In these cells a larger proportion of yolk globules stain than in a preparation from the entoderm. Also, there is more pigment in proportion to the amount of yolk. There is no pigment in the nuclei. Greenish "alpha" granules have not been seen to change into pigment granules.

5. Methylene Blue Preparations.

Methylene blue was used very few times. It did not react uniformly through the preparations. In some fields the dye would stain a few of the yolk globules. In such a group the granules on their surfaces and the pigment granules free in the preparation appeared to stain a deep blue. It was thought that these yolk globules that reacted to the stain may have been "beta" globules, and that the granules that were stained on their surfaces may have been "beta" granules. In the fields where the stain reacted, no colorless granules were seen.

6. Nile Blue Preparations.

Nile blue was used as a stain for the dissected fresh material from embryos of different ages, and as an intravitam stain. Nile blue, even when used in very dilute solutions, was apparently too toxic for normal growth. In different groups placed in the dye, from one-fourth to all of the specimens died in a few hours. Portions of entoderm, muscle and skin, dissected from stained specimens, were examined. The yolk globules stain differentially, some deeply, but most of them lightly. The muscles do not react readily to the stain. Granules, assumed to be "beta" granules, reacted to the stain. Within some groups of granules there seems to be a gradation of color from a lighter blue to an intense blue. The pigment granules, free in the preparation, stained a dark, intense blue. Melanin granules, from chromatophores in the skin preparations, did not react to the stain, or only slightly. By changing the focus some of the melanin seemed to have a greenish tinge. This may have been due to diffraction or slight adhering of the dye to the granules.

Fresh preparations from living embryos react to Nile blue very readily. Dilutions from 1:50,000 to 1:60,000 were generally used. The nuclei do not stain. Nearly all the yolk globules react to the dye, although some deeper than others. Pigment granules stain a very deep blue, so dark as to appear almost black. In some prepara-

tions there were free yolk globules that stained differentially. Deep blue granules were observed to be on the surfaces of some of the yolk globules and many of them exhibit gliding vibratory movement. This was most active on the smaller globules. Only one granule in these Nile blue preparations has been seen to leave a globule, and it then had the appearance of a free pigment granule. From the staining of the granules and yolk it seems that Nile blue stains the "beta" globules and "beta" granules. No colorless granules were observed.

7. PANCREATIN DIGESTION EXPERIMENT.

The undifferentiated entoderm was removed from several embryos by careful dissection, with particular attention not to include an undue amount of pigment from the skin. Portions of the tissue were placed in a solution of pancreatin, and digested in an incubator for a number of hours. Others were incubated in physiological salt solution alone. Portions of the mixture were removed at intervals of from six to forty-eight hours and examined, both unstained and stained, for evidences of production of granules by the action of digestive enzymes.

The appearance of the unstained digested preparations is very much the same as the unstained preparations examined immediately after removal from the body of the embryo. The yolk globules vary in size. There were some colorless granules on yolk surfaces, but there was apparently no increase in amount over normal conditions. Very few pigment granules were present and they were mostly free in the preparation. There was no apparent increase in number of pigment granules due to digestion. The pigment granules were most numerous in fields containing many small yolk globules.

The use of neutral red did not show an increase in amount of pigment. However, it appears that more of the yolk stains than in living preparations. The smaller globules seem to take the stain more readily than the larger ones. The excrescences and "beta" granules occur in very much the same way in incubated undigested as in digested preparations. In both preparations there was apparent differential staining of granules on the surfaces of globules from a lighter red to the dark red of the pigment granules.

Nile blue presents a similar picture. The majority of the yolk globules stain. The smaller globules stain deeper that the larger ones. More deeply stained granules occur on the surfaces of the smaller, deeply stained yolk globules. There was apparently no increase in number of granules. Sharlach R reacts in a similar way.

The pigment granules stain slowly to a deep red. The yolk stains differentially. The smaller globules stain more deeply than the larger ones. The granules on the surfaces stain deep red.

The pancreatin digestion experiment did not show an increase in amount of "alpha" or "beta" granules. It did show, however, that the action of the digestive enzymes on the yolk globules prepared more of them for staining. It appears that the action of pancreatin occurs most rapidly on the smallest globules.

8. Conclusions.

The work on the origin of the pigment shows the following: (1) presence of colorless granules in unstained preparations; (2) differential staining of yolk globules and of granules on their surfaces; (3) the pigment granules are formed in the cytoplasm; (4) the pigment does not come from the nucleus; (5) the differential staining of granules on the stained yolk globules indicates that the pigment is formed from substances in the cytoplasm, part of which may be liberated during the process of digestion of the yolk.

D. NATURE OF THE PIGMENT.

The pigment in the differentiating tissues of the embryos studied is not in sufficient quantity to permit separation from the tissues. For this reason a chemical analysis has not been made. Microchemical tests for the study of pigment in tissues are now being standardized, as shown by the work of Hueck (1912), Dolley and Guthrie (1918, 1921), Schmidtmann (1920) and others.

1. Staining Reactions in Fresh Preparations.

Various stains have been used on fresh tissue to observe the reaction of the dyes on the pigment. Neutral red, methylene blue, Nile blue, Janus green and the fat stains, Sudan III and Sharlach R, were used.

The pigment granules, yellowish brown to brown in natural condition, stain with neutral red, Nile blue and methylene blue.

In preparations from the older specimens the pigment from the chromatophores were frequently included. This pigment is easily distinguished from the pigment in the differentiating tissues by difference in size and color of the granules. The pigment in the chromatophores stain very slightly with neutral red and Nile blue and not at all with Janus green. This pigment is melanin elaborated by specialized cells. Fat stains do not stain it.

The fat stains, Sudan III and Sharlach R, were used primarily in an attempt to determine the relations of the pigments to fat. The results were the same in both cases, although Sharlach R was the more commonly used. Preparations were stained similarly as with the other stains. Yolk globules stain differentially; some a very light red; others a deep red. Portions of some globules stained deeper than the remainder of the globules. The majority of pigment granules occur about the deeper stained yolk globules. The granules on the surface of the deeper-stained globules seem to stain with approximately the same intensity as the globules. The pigment granules, free in the preparation, appear either to have actually stained or the stain has saturated the surrounding tissue, and thus causes the granules to appear darker. Biondi's stain, containing osmic acid, was used, with the result that the pigment granules are blackened by the osmic acid and retain the color through paraffin imbedding.

2. Staining Reactions in Sectioned Material.

The observations on the distribution of the pigment have been on sectioned material stained with various histological stains. Such stains as eosin, erythrosin, orange G, and Lyon's blue do not react with the pigment. Other stains appear to act very weakly at times. This was observed with methylene blue, neutral red and Nile blue. Such reactions are very slight. They appear to be a physical combination only. The pigment does not stain with alum-carmine and Janus green. It seems to be affected by imbedding in paraffin after alcohol-xylol treatment as shown by the weaker staining reactions with neutral red and Nile blue.

3. Solubilities.

The routine procedure has been to fix tissue in ordinary fixing solutions, carry it up through graded alcohols and clear in cedar oil or xylol and imbed in paraffin. The pigment does not disappear from the tissue. More powerful fat solvents were used than alcohol, such as chloroform, acetone, ether and xylol. After prolonged treatment of twenty-four hours or longer the pigment did not disappear, or only very slightly. Dolley and Guthrie (1921) state that lipochrome or fat-holding pigment will disappear from tissue after treatment of one-half hour in absolute alcohol. There was no appreciable disappearance of pigment after such treatment, even if tissues were kept in absolute alcohol from two to four hours.

4. VARIOUS OTHER TESTS.

Hydrogen peroxide and ferric chloride oxidized the pigment and changed it to a colorless state after a few hours. The pigment of the chromatophores also is bleached by the oxidizing agents.

The pigment gives a negative iron reaction to ferrocyanide of potassium followed by acid alcohol. This is true also of the melanin of the chromatophores. The pigment is insoluble in dilute acids, alkalies and ammonia water.

5. SUMMARY AND CONCLUSIONS.

Since the pigment reacts with fat stains, it seems to be necessary to class it with fat-holding pigments. The fat solvents, however, which dissolve fat-holding pigments, called lipochromes, do not remove this pigment appreciably, so that in tissues prepared with histological methods it presents characteristics of melanin.

E. DISAPPEARANCE OF PIGMENT.

The study of relation of pigment to differentiation has been confined to the early swimming and younger stages, because during this period blood vessels have not entered the myotomes and central nervous system. Soon after the embryo swims the blood vessels are in close proximity to the brain and rapidly come in contact with it and later with the spinal cord. Before the feeding stage blood vessels can be seen between the myotomes. This added factor of blood vessels connected with the tissues to serve in nutrition and excretion necessitated a study of its effect on the amount of pigment.

In order to determine whether this pigment is distinctly of embryonic nature, tissues from several adult *Amblystoma* have been examined. It was found that the pigment had entirely disappeared from skeletal muscle. The same is true also of adult muscle of *Rana pipiens*, *Necturus* and *Bufo*. Serial sections have been studied of several embryos older than the early swimming stage to observe the time and method of the disappearance of the pigment.

In an embryo approximately two days older than the early swimming stage the pigment has the same general distribution as described for the early swimming stage. An exact count of the number of pigment bodies in muscle at this time has not been made, but there is no apparent difference from the younger stage. Four embryos were observed that had just attained the feeding stage. At this time blood vessels can be seen between the anterior myotomes. In these myotomes the relations of the pigment in the cells

have changed. Many of the pigment bodies are irregular in shape and appear to be breaking up. The pigment bodies are not confined to the middle third of the myotome longitudinally. A few are now situated in the end region between the nuclei and the end of the myotome. Very frequently these bodies were situated close to the blood vessel. In a few instances pigment bodies were seen in a blood vessel. Although there appears to be a migration of pigment bodies or fragments of pigment bodies toward the blood vessels this occurs comparatively slowly.

In embryos two, seven and fifteen days older than the feeding stage there is progressively less pigment in the myotomes. The pigment begins to disappear first in the anterior myotomes, and its disappearance proceeds caudad. In serial sections of portions of myotomes in *Amblystoma* larva 38 mm. in length, a few pigment bodies occur near the ends of some nuclei. Pigment granules are occasionally seen near the pigment bodies. The pigment is not abundant. Its complete disappearance occurs between this age, which is approximately six weeks after feeding, and metamorphosis. The pigment seems to be removed from the myotomes through the blood. This phase of the problem demands further study.

DISCUSSION.

In this discussion of the writer's results in the light of the work of other investigators, the pigment, with which this paper is concerned, will be considered with reference to (1) its origin, (2) its nature, and (3) its significance.

A. ORIGIN OF THE PIGMENT.

The pigment described in differentiating tissues of *Amblystoma* occur in cells that are particularly adapted for study of pigment formation. Not only is this true for study of the origin of the pigment, but also for the study of intracellular metabolism, since each cell from the beginning has food, in form of yolk, sufficient for growth, differentiation and embryonic function. During the period under consideration there are no blood vessels in the tissues concerned to serve in nutrition or excretion. During this period of development, before the blood vessels enter the tissues, each cell of the tissues must produce all necessary enzymes for its absorption of the yolk and for the metabolism of differentiation and proliferation. Some enzymes or hormones must prepare the yolk for as-

similation similarly to the process of digestion; other enzymes must aid in the physiochemical reactions of metabolism.

Since these cells are thus adapted for study of intracellular digestion, assimilation and metabolism, they have been particularly examined for evidence concerning the place and mode of formation of the pigment that occurs within them.

Various workers, such as Von Szily (1911), Schultz (1912), Wagner (1910), and Dyson (1911), hold to the view that the pigment arises in nuclei. The writer finds no evidence that the pigment is produced by the functional activity of the nucleus. No pigment has been observed either in the nucleus or imbedded in the nuclear membrane. Observations in the younger cells, especially in the muscle sections, show that pigment granules occur on the surface of the nuclei, but there is no evidence that it came from nuclear material. No extrusion of granules from the nucleus, colorless or pigmented, have been observed. In no phase of the work have nuclei in pigmented cells appeared shrunken or degenerative.

According to Child (1915) "undifferentiated protoplasm is protoplasm reduced morphologically to its lowest terms." The embryonic cells under consideration are morphologically simple. The most conspicuous structures in the cytoplasm are the yolk globules, which are so numerous that they hinder the observations of the relatively permanent structures that are formed in the cells. Observations have been made on both unstained and stained preparations to observe the possible presence of chromogen and its transformation into pigment. The two possible sources for the chromogen are (1) mitochondria, and (2) other substances in the cytoplasm.

Some authors, such as Champy (1911) and Luna (1913), have described the formation of pigment from mitochondria. Mitochondria are not present in large numbers in embryos of *Amblystoma*. This was noted by Coghill (1915) and was verified in the present series of experiments by the use of Janus green. This dye, which is a specific stain of mitochondria, stained certain granules on the surfaces of yolk globules, as was noted in the observations. These granules may develop into mitochondria, but this has not been positively demonstrated. At no time has the stain reaction of these granules and of mitochondria suggested that mitochondria develops into pigment.

The chromogen of the pigment seems to arise in the cytoplasm as colorless granules on surfaces of yolk globules. These colorless granules are observed in the unstained preparations, but are not observed

in preparations stained by various dyes. The use of neutral red, especially, shows a differential staining of granules on yolk globules from a light to a dark red. The darker granules when they detach themselves from the yolk cannot be distinguished from pigment granules free in the preparation. The size, position and staining reactions of the granules on the surface indicate that the colorless granules are the chromogen of the pigment granules and that they arise on the surfaces of yolk globules. This does not exclude the possibility of the formation of the chromogen in the cytoplasm apart from the yolk globules. In fact such an origin is suggested in the serial sections of the youngest embryos, where the pigment was observed particularly in the vicinity of the nuclei. In those cells the physiological activities naturally occur near the nucleus and the action of the cell ferments converts the chromogen into pigment. Later the chromogen probably is formed faster than it becomes pigmented as suggested by Smith (1920), thus explaining the presence of colorless granules. Smith notes that the pigment in the embryonic chick's eye develops first as a colorless chromogen and later becomes colored.

The nature of the chromogen is debatable. As has been cited in literature, pigment may come from a number of substances. In the production of melanin, different workers such as Bertrand, Gessard, Durham, and Block have shown that such compounds as tyrosin, tryptophane, 3-4 dioxyphenylalanin, epinephrine, occur in tissues in sufficient quantities for the production of the pigment. The cell ferments are formed by the nucleus. Whipple (1921) concludes that substances containing the pyrrol group are essential for formation of hemoglobin and bile pigments. Melanin on decomposition yields compounds containing the pyrrol group. It is probable that substances such as tyrosin and pyrrol derivatives arise during the differentiation of the cells in the embryo of *Amblystoma*.

As the young cells contain a nucleus, relatively undifferentiated cytoplasm and food in form of yolk, the substances that enter into the formation of the pigment must be produced through the digestion of the yolk or as a result of metabolism. Or they may be formed by a combination of both these processes. As has been noted in the observations the pigment is formed in the differentiating tissue. So that whenever, in the process of differentiation, substances capable of forming pigment are produced in excess chromogen might be the result.

Some of these substances no doubt are formed from the yolk during its digestion. The differential staining reactions of the yolk globules by neutral red and Nile blue show that physiological processes occurring on the surface of the yolk produce or liberate certain substances that stain more readily than the yolk globule as a whole. The digestive enzymes present may break up the yolk into the amino acids which in turn are used in building up the protoplasm of the cell. As the yolk is the same for all cells, then those cells which are differentiating the most rapidly, may not have use for all the constituents of the yolk and it would be these substances that are produced in excess which may enter into the formation of chromogen. Since during the early embryonic period in *Amblystoma* there are no blood vessels in the muscles and nervous system these substances must remain within the cells that produce them.

The writer has made no attempt to determine the chemical composition of the yolk. McClendon (1909), working on centrifuged frog eggs, was the first to attempt the chemical analysis of yolk. He concludes that it consists of some fat, considerable lecithin and a large quantity of protein. He considered the protein to be a nucleo protein as it was rich in phosphorus. Jenkinson (1914) reports on the composition of yolk of frog's eggs, and considers the protein to be a globulin or nucleo-protein and that a purine base, which probably is xanthine, can be obtained. He points out the difficulties of chemical analysis even in quantities of the yolk and the impossibility of such analysis of a single egg. Nevertheless these investigators have shown the presence in the yolk of proteins that can be broken up into amino acids and synthesized into body protein. It is believed, therefore, that the yolk of *Amblystoma* is of such composition that pigment-forming substances can be derived from it during its digestion.

The writer's observations upon the origin of this pigment have been performed on the epithelial cells of the skin rather than upon those of the entoderm, since the ectodermal cells show a greater degree of differentiation. The metabolic rate is presumably higher in cells that are differentiating more rapidly, and it is in these rapidly differentiating cells that the pigment increases at the most rapid rate.

Whipple (1921), after a review of the various factors involved in the metabolism of bile pigments, has evolved a new working hypothesis as an explanation of the metabolism of pigment. The es-

sential feature of this hypothesis is the "pigment complex," which he describes as a "group of substances which are essential parts of the mature body pigments." Whipple believes that experimental evidences show that the "pigment complex" is formed from substances obtained from (*a*) food, (*b*) body cells, and (*c*) hemoglobin. Formed from the "pigment complex" are hemoglobin, bile pigments and, possibly, urobilin and urochrome. Whipple (1921) holds that the pyrrol group seems to be the essential factor in the chemical composition of the substances in the "pigment complex."

In these cells under consideration, it has already been suggested above that the pigment may come from substances derived from the food. It is also probable that other substances are formed during metabolism which enter into the formation of this pigment. These substances formed by metabolic processes could, if there were vascular and excretory systems, be carried out of the tissues and either utilized by different organs of the body or excreted, but in the absence of circulatory systems the substances that are probably comparable to Whipple's "pigment complex" are necessarily retained within the cell.

The waste products from metabolism may be formed in either the anabolic or catabolic phase of metabolism. The anabolic phase involves the building up of complex elements out of more simple molecules. Macleod (1922) states that chemical transformations that occur in the cell are difficult to obtain in the chemical laboratory. He says: "For in the cell these chemical transformations are capable of being guided to a very remarkable degree of nicety so as to produce intermediate products that are used for some special purpose, either by the cell that produced them, or after transformation by the blood, etc., by cells in other parts of the organism." Such products, which Macleod calls intermediate products, the writer considers, enter into the formation of pigment in *Amblystoma* embryos. In this inert condition they are retained within the cell until the tissues are penetrated by the vascular system.

Many workers have shown that waste products of many tissues have been formed into pigment, and recently Whipple (1921) has shown that substances from the breaking of the body protein enter into the "pigment complex," from which bile pigments and hemoglobin are formed. Thus in the differentiating tissues of *Amblystoma* embryos substances formed during the catabolic phase of metabolism probably enter into the formation of the pigment. As soon as the tissue begins to differentiate, the building up and tearing

down of the protoplasm begins. The substances from the wear and tear of tissue cannot be eliminated, and thus enter into the formation of the pigment.

The group of substances mentioned above that are formed during the differentiation and embryonic function of the tissues under consideration might well be regarded as a "pigment complex." By "pigment complex" is meant in this connection a group of substances that are formed in the differentiating tissue from three probable sources, as follows: (1) Excess of substances formed during digestion of the yolk that are not needed by the tissue cells; (2) intermediate products of building up of the protein during the anabolic phase of metabolism; and (3) products of breaking down of the cell protein. It is considered that as the substances accumulate they are converted by activity of the cell into pigment. The colorless chromogen as precursor of this pigment probably is laid down, then the color develops in it. This would account for the presence of the colorless granules which appear to be changed into pigment.

B. NATURE OF THE PIGMENT.

The pigment in differentiating tissues of embryonic *Amblystoma* does not occur in sufficient amounts to allow isolation from the tissues. A chemical analysis of it, therefore, could not be made. In order to determine the nature and classification of the pigment it was necessary to depend upon microchemical methods.

This pigment is a waste product of normal metabolism and has many characteristics in common with the waste pigments found in the human body. Lubarsch (1902) and his pupil Sehrt (1904) found a waste pigment in different parts of the human body which they called lipochrome. Lubarsch advanced the theory that the lipochrome is a byproduct of metabolism. Sehrt observed that the pigment in the tissues nearly always gave strong reactions to fat stains in fresh preparations and only weakly or not at all after treating with alcohol and imbedding in paraffin. He does not recognize the possibility to two kinds of pigment being present. Other workers have noticed the fat-staining characteristics and partial solubilities of waste pigments. Hueck (1912) found that "wear and tear" pigment reacts to Sudan III, Sharlach R and Nile blue. He believes the reaction of Nile blue is characteristic for fatty acids. Hueck suggests the possibility that "wear and tear" pigments are products of fat metabolism. Ciaccio (1915), working on auto-oxidation experiments of lipoids found that unsaturated fatty acids are oxi-

dizable and undergo important changes of color, consistency, solubility properties, and reactions towards alkalies and acids, while they retain their natural characteristics to react toward fat stains. He distinguishes two types, one derived from phosphatids which react weakly towards fat stains, and the other derived from fatty acids which react intensely with Sudan III and Sharlach R. Schmidtmann (1920), after extensive study of sectioned material from brown atrophy of the heart, concludes that the pigment which is present in sectioned material is melanin, because it does not differ in staining reactions and solubilities from melanin. Schmidtmann does not believe that it is a metabolic product of fat, but that the staining of the pigment granules in fresh tissues indicates that there is a mixture of fat with the pigment of more or less accidental nature and that there is a connection between nutrition and fat content of pigment.

Recently the biochemical and physiological studies of Palmer and Eckles (1914) and Palmer (1919) have demonstrated that the plant pigments carotin and xanthophyll are widely distributed in animals and are intimately connected with animal metabolism. The carotinoids (carotin and xanthophyll) were found to be identical with the pigments in blood serum, skin, corpus luteum, milk, and in the body fat of the cow, horse and hen. There is a remarkable species difference. Color is produced after feeding foods containing carotinoids, but it disappears if deprived of the pigments.

Dolley and Guthrie (1921) find that the pigmentation of brown atrophy of the heart consists of not only the lipochrome usually ascribed to it, but also a melanin, which is a waste product. They believe that the lipochrome is derived from plant carotinoids and carried to the heart by the blood, while the melanin is "an endogenous pigment derived from disturbed metabolic processes."

Although it was formerly held that waste pigments are lipochromes of animal origin, recent workers, such as Palmar and Eckles, Schmidtmann, Dolley and Guthrie, have shown the presence of two waste pigments, one a melanin of endogenous origin and the other a lipochrome derived from the plant carotinoids in the food.

The reactions of the pigment in the differentiating tissues of *Amblystoma* in sectioned material agree with the characteristics of melanin. It stains only slightly with fat stains or basic dyes. It is iron free. It persists in sectioned material after imbedding in paraffin. A very small portion, if any, of the pigment is dissolved by fat solvents. The objections to classifying the pigment as

melanin are two, namely, (1) the staining reactions in fresh preparations, and (2) the presence of the pigment in tissues which do not ordinarily elaborate melanin.

Various stains were used in fresh preparations to determine the nature of the pigment. It has been already noted that the colorless granules, which are believed to be the chromogen, and the pigment granules, react positively to Sudan III, Sharlach R, Nile blue and neutral red. This indicated that the granules, chromogen and pigment, are either related to fat metabolism or intimately mixed with the fat or fatty acids in the cytoplasm.

It is plausible that metabolic products of the fats and fatty acids form colored compounds, according to the work of Ciaccio. The yolk contains fat which undoubtedly is used in the reactions of the metabolism. If intermediary products of metabolism of fat or end products formed that are not needed in the cell they may contribute to the chromogen of the pigment. That pigments in animals are formed directly from fats has so far not been proven.

The writer is of the opinion that the mixing of the fats and fatty acids of the cell in a physical manner with the pigment accounts for the positive staining reactions with fat stains in fresh preparation. That this might be possible has been suggested by various authors, such as Dyson (1911), Hueck (1912), and Schmidtmann (1920). The writer's study of the relation of fats to the pigment was not carried further than the use of the fat stains. The same pigment granules that stain with fat stains in fresh preparations react only very slightly to them or to basic dyes in sectioned material. This could easily occur as the alcohol used in dehydration of the tissue would extract the fat.

The writer does not believe that the waste pigments should be classified as melanin without further study. Schmidtmann (1920) and Dolley (1921) state that the pigment in brown atrophy of the heart is melanin because it gives many of the microchemical reactions of melanin. If this pigment is considered melanin it destroys the general conception of the majority of workers that melanin is a product of specialized cells produced for a particular purpose. It seems more reasonable that the pigment formed in the heart from metabolic products by the activity of the cells would not be found there if the interchange of products of the cell and substances in the blood plasma were normal. If these same substances that form pigment where the circulation is impaired were normally carried from the tissues, then they might be regarded as substances that occur

in the "pigment complex" of Whipple. The writer believes that in the differentiating tissues of *Amblystoma* the substances which form the pigment are comparable to those substances in Whipple's "pigment complex."

It may be shown in the future that the substances that are used in the formation of melanin are at least a part of the substances in the "pigment complex." Further work is needed on pigment metabolism to show the relation of the substances from which melanin is formed to those substances from which hemoglobin and bile pigments are derived.

The waste embryonic pigment in *Amblystoma* is derived primarily from metabolic products of protein metabolism and food, and is intimately mixed with fats, probably physically and not chemically. This pigment is comparable to bile pigments, since it is a waste product. In certain other features it resembles melanin, but it cannot be unconditionally classified either as melanin or lipochrome. In certain specialized cells it may be retained and further differentiated into melanin in definitive organs.

C. SIGNIFICANCE OF PIGMENT.

Any classification of this embryonic pigment would mean very little if the relation of the pigment to the tissue were not considered in the terms of the life of the whole organism. In other words, the significance of the pigment can be understood only as it is considered in the light of the principles underlying the development of the embryo as a whole.

A few investigators of different amphibian eggs and embryos have offered suggestions concerning the probable significance of the pigment. Bellamy (1919), from his studies, said: "It may be noted here also that pigment appears more densely in the most active regions of the egg where other evidences indicate that oxidations are proceeding more rapidly than elsewhere. In other words, the density of pigmentation seems to be an expression of the rate of at least certain oxidations occurring in that region." He suggests that increased pigmentation is related to regions where cell division is most rapid. Jordan (1893), from observation on the newt, suggested "that the pigment marks physiological activity, and that the less heavily pigmented cells of the ventral wall of the archenteron owe their relative lack of pigment to more sluggish metabolism attendant upon less rapid cell division." King (1902), working on the egg of *Bufo lentiginosus*, doubts the probability that pigment increases with rapid cell

division, for, she says, "It is certainly true that the large yolk cells in the egg of *Bufo* divide less frequently than the cells in the upper hemisphere, but there is no evidence that the deeply pigmented cells of the outer surface of the upper hemisphere or of the dorsal wall of the archenteron divide more rapidly than the cells that are found between them."

While the observations of the above-mentioned investigators were incidental to their work on other major problems, in the writer's studies which concern pigment particularly, it is found that pigment makes its appearance as a result of "oxidation processes" or "physiological activity" in early embryos of *Amblystoma*, but it is obvious that there are more remote factors in pigment formation than simply "oxidation" or "physiological activity" as conceived by Bellamy, Jordan and King. These factors have to do with the difference between differentiation metabolism and proliferation metabolism, for these observations demonstrate that pigment accumulates in regions of differentiation, but does not accumulate in regions of proliferation. While both these processes involve "oxidation" and "physiological activity," the accumulation of pigment in connection with the one and not with the other shows that the physiological processes must be fundamentally different in the two cases.

In differentiation there is a building up of structures that render the protoplasm more complex. Accompanying the building up of these relatively permanent structures of the cell, by which differentiation is determined, is a certain amount of wear and tear in the protoplasm itself, and this must involve both oxidation and reduction processes. Among the products of these processes of "wear and tear" would be the mother substances of pigment. The metabolism of proliferating cells, however, does not result in the building up of complex, relatively permanent structures in the protoplasm. On the contrary, according to Child (1915) mitosis involves dedifferentiation in the cytoplasm. Proliferation, therefore, must lack the "wear and tear" processes that are characteristic of differentiation, and for that reason lacks the function of pigment production.

Bearing upon the question of the relation between differentiation and dedifferentiation, Child (1915) says: "Cells which divide rapidly do not undergo any great degree of differentiation, and the cells which resume division after undergoing differentiation first undergo a greater or less degree of dedifferentiation." Therefore, in embryonic cells which are undergoing rapid proliferation, such as

those of the ventricular region of the central nervous system of the embryo, the slight differentiation metabolism which may be taking place must be counterbalanced by the predominating processes of dedifferentiation. In such relations differentiation and dedifferentiation must be conceived as antagonistic processes, and the antagonism between these processes must be conceived as involving all that has to do with the substances of the "pigment complex." Any substances of the "pigment complex" that may arise during the resting phase of the cell might become dedifferentiated in the following mitosis. This view receives support from Child's (1915) work on flat worms, in which he shows conclusively that in dedifferentiation highly differentiated tissue structures actually disappear.

The application of this principle of antagonism to the development of *Amblystoma* during the period under consideration explains why mitosis occurs very rarely or not at all in the myotomes which are undergoing rapid differentiation and in the rapidly differentiating regions of the central nervous system. It renders intelligible, also, the polarity of the amphibian egg with reference to pigmentation. Bellamy (1919) considers that the polarity of the frog's egg is determined by the relation of the egg to the blood supply. He says: "This polarity is marked by the localization of most of the protoplasm in the pigmented hemisphere, by the eccentricity of the nucleus, and by the distribution of the pigment." He holds, also, that "local increase of activity results in the formation or the increase of pigment, and local decrease in activity results in diminution of pigment." However, the results of the writer's studies show conclusively that the term "activity" is wholly inadequate to indicate the real nature of the processes under consideration. The conception of polarity as the localization of differentiation metabolism renders the problem of polarity of the amphibian egg intelligible from the point of view of the writer, for yolk is produced in the cytoplasm through differentiation metabolism, and it is necessary that this process take place in the region of oxygen and food supply. But this differentiation metabolism involves the production of the substances of the pigment complex, and in the presence of abundant oxygen they readily become transformed into pigment. The localization of pigment in the egg is, therefore, determined by external conditions as Bellamy proposed, but its origin is specifically involved in the process of differentiation.

CONCLUSIONS.

1. The pigment that is characteristic of amphibian embryos arises in the cytoplasm from colorless granules which are considered to be chromogen and which represent a pigment complex made up of (1) substances that arise in excess from digestion of the yolk, (2) intermediary products of anabolic phases of metabolism, and (3) products of catabolic phases of metabolism.

2. This pigment cannot be unconditionally classified as either melanin or lipochrome, but is regarded as a waste pigment comparable to the bile pigments.

3. This embryonic pigment is an indicator of metabolism that is characteristic of tissue differentiation, as opposed to metabolism that is characteristic of all cell proliferation.

4. In the light of pigment formation, differentiation metabolism and proliferation metabolism are regarded as fundamentally antagonistic processes in development.

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DESCRIPTION OF FIGURES AND PLATES.

REFERENCE LETTERS: *pb*=pigment body. *pg*=pigment granules. *n*=nucleus. *yg*=yolk globule. *s*=strands of pigment granules.

FIGURES I, II, III, IV.

FIGURE I. An optical projection of the pigment bodies in a nonmotile embryo as viewed from the side of the (*a*) third myotome, (*b*) tenth myotome.

FIGURE II. Projection of an early embryo similar to figure I, except in addition it contains the projection of (*c*) the eighteenth myotome.

FIGURE III. Projection of a coil-stage embryo similar to figure II.

FIGURE IV. Projection of an early swimming-stage embryo similar to figure II, except that (*d*) represents the twenty-fourth myotome.

FIGURES I, II, III, and IV.

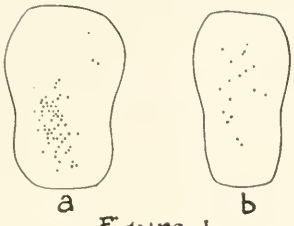


Figure I

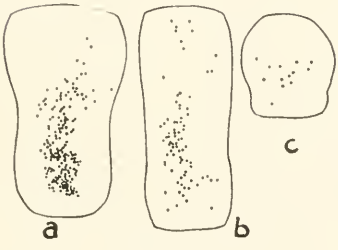


Figure II

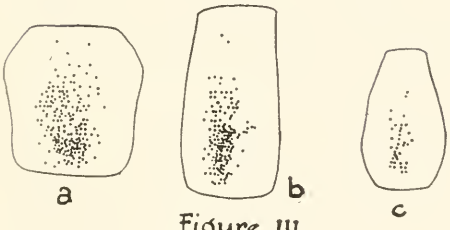


Figure III

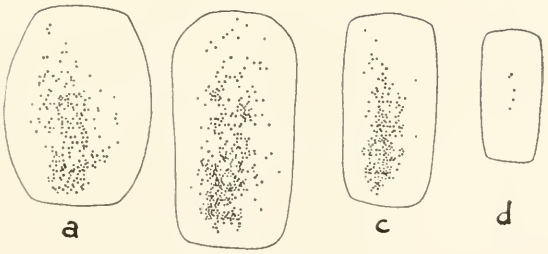


Figure IV

FIGURE V.

Figure V is a reproduction of drawings of sections taken at different levels of the central nervous system of the various stages, noting the location of the areas of abundant pigmentation by stippling on one side only. Row I was made from a nonmotile-stage embryo; row II from an early flexure-stage embryo; row III from a coil-stage embryo; row IV from an early swimming-stage embryo. Column 1 was taken at the level of the V root; column 2 at the level of the VII root; column 3 at the level of the IX root; column 4 from the spinal cord at the level of the third myotome; and column 5 from the spinal cord at the level of the eighth myotome.

FIGURE V.

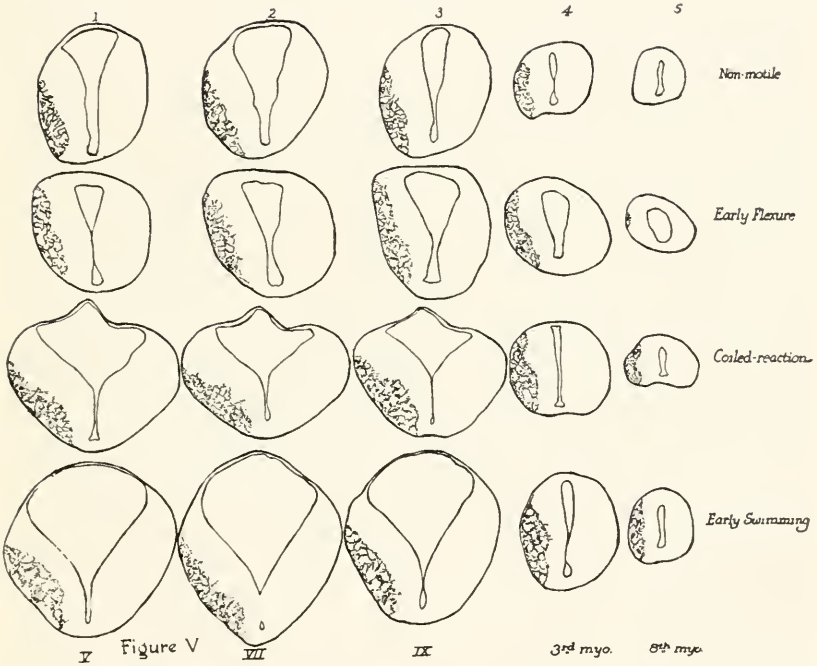


Figure V

PLATE XII.

A photomicrograph (× 800) of a frontal section of the third myotome taken through the middle, dorsoventrally, of an embryo of the early swimming stage, fixed in Bouin's solution and stained with Janus green and neutral red. Pigment granules appear rather indistinctly about the yolk globules and the nuclei. The pigment bodies are distributed chiefly near the middle of the myotome longitudinally. They appear as perfectly opaque objects. Four of them lie near the middle of the figure. Two others, smaller than those in the middle of the myotome, lie in the upper part of the plate, one to the left and the other to the right. The striations of the myofibrils accurately represent the degree of differentiation in the contractile tissue of the myotome at this stage in development.

PLATE XII.

PLATE XIII.

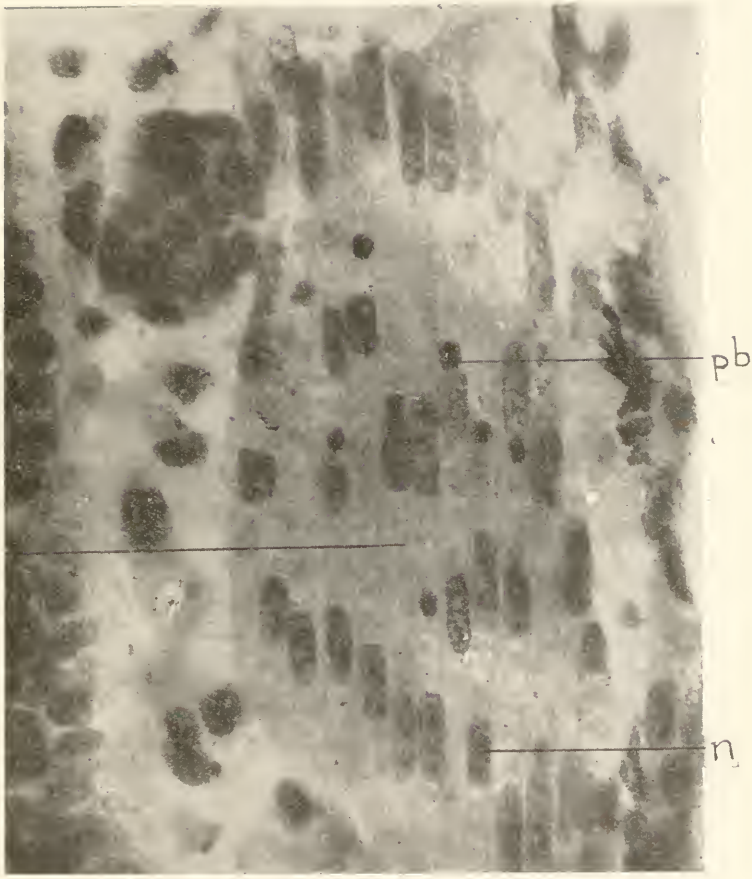
A photomicrograph (× 850) of a section taken from the dorsal portion of the tenth myotome of a nonmotile stage embryo. The cells are radially arranged about the center of the myotome. The pigment granules occur in the central part of the myotome. S represents strands of granules extending from ends of nuclei toward the center of the myotome. Yolk globules (yg) are faintly visible and some have pigment granules on their surface. The embryo was fixed in Bouin's fluid and stained with neutral red and Janus green.

PLATE XIII.

PLATE XIV.

This is a photomicrograph (× 440) of a section in frontal plane of the tenth myotome of an embryo of the early swimming stage. The section was taken from the dorsal portion of the myotome. The fixative was corrosive sublimate and acetic acid and the stain was alum carmine and Lyons blue. No pigment granules are visible at this magnification. A row of nuclei can be observed at either end of the myotome. The pigment bodies (pb) are mostly located near ends of nuclei.

PLATE XIV.



THE UNIVERSITY OF KANSAS
SCIENCE BULLETIN

Vol. XVI.] March, 1926. [No. 6.

On a Nearly Complete Lizard Skull from the Oligocene of Nebraska.

CHARLES W. GILMORE,
Curator of Vertebrate Paleontology, United States National Museum.

THROUGH the courtesy of Mr. Handel T. Martin, assistant curator in charge of the Paleontological Museum of the University of Kansas, I have been given the privilege of studying and describing the most perfect cranium of an extinct anguid lizard that has yet been found in North America.

The specimen is practically free from distortion, and consists of the essentially complete skull and lower jaws, articulated with the anterior cervicals and with much of the protective osseous covering of both head and neck preserved *in situ*. The value of the specimen has been greatly enhanced by its skillful preparation, a very delicate piece of work, accomplished after many days of close application on the part of Mr. Martin, who is to be congratulated on having so successfully executed this difficult task. The specimen was found in the Oligocene deposits of Hat Creek Basin in western Nebraska, by Mr. E. Raymond Hall, a member of the Kansas University Paleontological Expedition of 1922, led by Mr. Martin.

In 1873, on rather fragmentary materials, the late Prof. E. D. Cope[1] established the genus and species *Peltosaurus granulosus*, a lacertilian reptile from the Oligocene deposits in northeastern Colorado. Like many other extinct forms described from fragmentary specimens, *Peltosaurus* has stood for half a century without further knowledge of its skeletal characteristics. It was, therefore, of great interest to find that the Kansas specimen is referable to this little known genus and species.

1. Paleont. Bull., No. 5, 1873, p. 5.

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In the course of a monographic study of the lizards of North America, on which I am now engaged, all available Sauria materials found in North America have been assembled in the United States National Museum, and among these are a half dozen skulls of *Peltosaurus*, but the Kansas University specimen is the best skull of the genus that has yet been discovered. It supplements the others so fully that from a study of all it has been possible to work out the complete details of the skull structure, as well as much concerning the arrangement of its scutellation.

Since a full description of the cranium will be published in my forthcoming monograph, it seems unnecessary to here call attention to any but the more interesting features.

Viewed from above, the skull is triangular, broad behind, with a bluntly rounded muzzle. It has a greatest length from the tip of the nose to the posterior end of the squamosal of about 48 millimeters, a greatest width of about 28 millimeters, and greatest vertical height with the lower jaws of about 19 millimeters.

Viewed from the side (see Pl. XV, Fig. 1), the skull is of moderate height, with the top flattened, and the nose strongly inclined. It is quite evident that in life the top and sides of the entire head were almost completely covered by symmetrical osseous scutes, whose outer surfaces have a granular ornamentation. Many of these scutes are missing, especially those from the sides. The skull is of the normal lacertilian type, with a single parietal, perforated by a pineal foramen. The frontal is single, the nasals distinct, and premaxillary single. A slender columelli cranii is present, and a nearly perfect palate. It is the first specimen of Peltosaurus to have the palate preserved, and this adds greatly to the importance of the specimen. It is shown that the pterygoids and palatines are well separated along the median line, and that there was a single row of small teeth on the pterygoid.

The dentition is pleurodont. The dental formula is: Maxillary teeth, 16; premaxillary, 8; and dentary, 21. The teeth are closely placed with subcylindrical shafts. Their apices are bluntly wedge-shaped, with the cutting edge in the longer axis of the jaw. The inner bevel of the crowns is much longer than the outer, and its surface is sometimes worn concave. Under the glass are seen delicately parallel wrinkling that runs at right angles to the cutting edge on the crown surfaces.

The osseous scutes of the body are arranged in rows encircling the

neck, one row overlapping the other. The scutes on the head are symmetrically arranged, and an interoccipital is constantly present.

All of the above features are in accord with its inclusion in the family Anguidæ to which *Peltosaurus* is now definitely referred. The important structural features of the genus and species *Peltosaurus granulosus*, observed for the first time in the Kansas University specimen, are as follows: Correct profile and contours of the skull; complete structure of the palate; presence of pterygoid teeth; presence of a tabulare; presence of a columelli cranii; complete brain case; arrangement of dermal scutes of the neck and throat.

EXPLANATION OF PLATE XV.

FIGS. 1 to 3. Skull and dermal scutes of *Peltosaurus granulosus* Cope. No. 1208, Kansas University Museum. Reproduced from photographs. All figures about twice natural size.

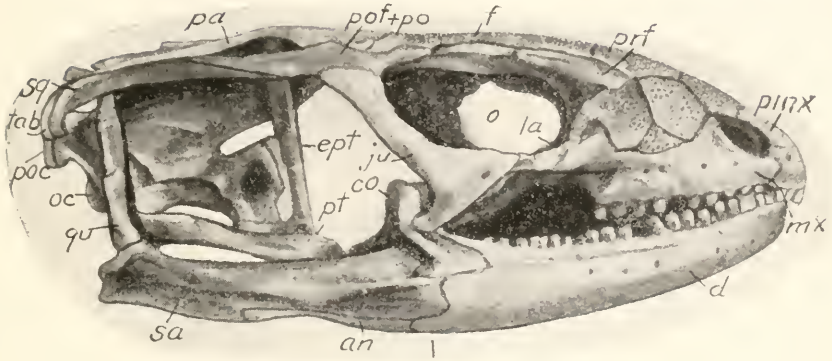
FIG. 1. Skull and lower jaws; viewed from the right side.

FIG. 2. Skull viewed from above.

FIG. 3. Dermal scutes covering neck; superior view. This block joins the back of the skull.

a, Anterior end of block which joins back of the skull; *an*, angular; *co*, coronoid; *d*, dentary; *ept*, epipterygoid; *f*, frontal; *ju*, jugal; *la*, lachrymal; *mx*, maxillary; *o*, orbit; *oc*, occipital condyle; *pa*, parietal; *pmx*, premaxillary; *poc*, paraoccipital process, *pof*+*po*, postfrontal + postorbital; *prf*, prefrontal; *pt*, pterygoid; *qu*, quadrate; *sa*, surangular; *sq*, squamosal; *stf*, supratemporal fossa; *tab*, tabulare.

PLATE XV.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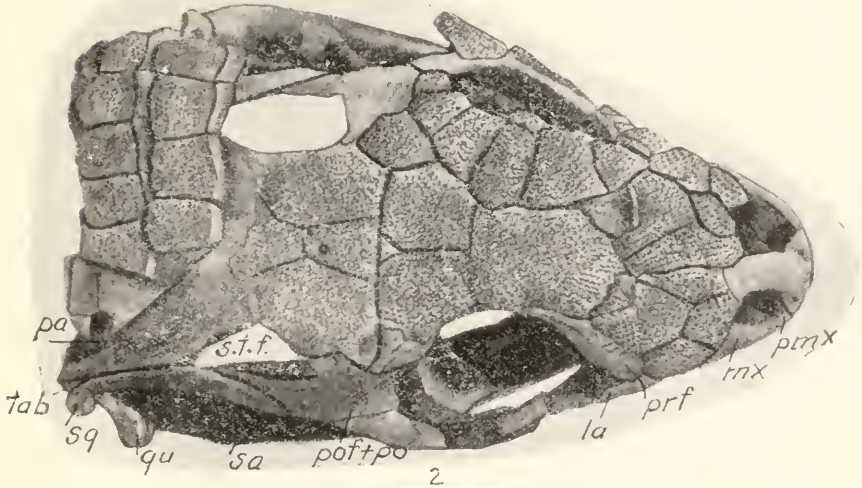
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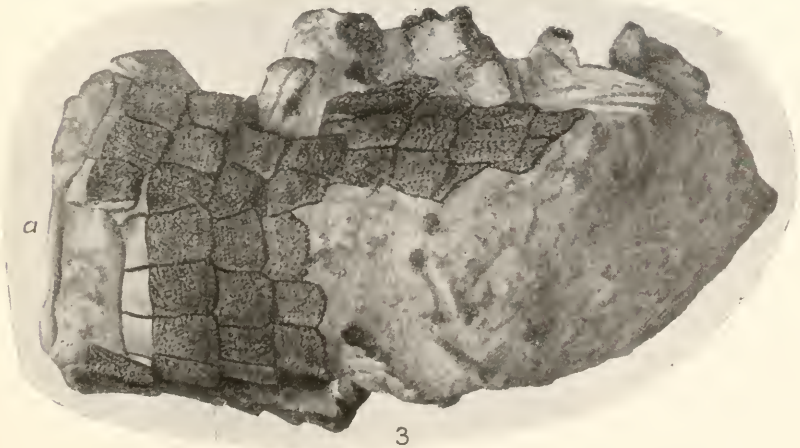
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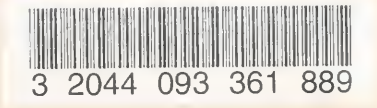
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